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Assessment of Rabbit Ovarian Reserve and Folliculogenesis by E-cadherin Plasma Level Estimation

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Abstract

Background The primary reproductive organs in females are the ovaries, which produce hormones like estradiol and progestins as well as release mature ova. The ovary is made up of two layers; the outer layer, known as the cortex and the medulla is the inner layer. E-cadherin is a single-span transmembrane glycoprotein with five repetitions and a cytoplasmic domain. It plays a variety of roles, including ensuring tissue integrity and resistance to stretching, cell signaling, regulation of cell proliferation, apoptosis, survival, and carcinogenesis.

Objective To assess E-cadherin level as predicting parameter for ovarian reserve.

Methods Female's rabbits (*Oryctolagus cuniculus*) of age (10 to more than 108 weeks), weighting 600-3000 grams were randomized into two groups: group A (25 sample, young group), group B (25 sample, old group). All groups' animals prior to euthanasia, blood was collected from the heart for assessment of plasma level of E-cadherin and then euthanized the animals. Specimens of ovaries were placed on standard glass slides for Hematoxylin and Eosin stain for demonstration of the cellular components of the ovary and follicles at various developmental stages.

Results The current study showed significant differences of plasma E-cadherin levels (P = 0.003), primordial follicles count (P <0.001) and antral follicles count (p<0.001); however, there was no significant difference of ovarian weight (P = 0.457) between group A and group B right ovaries respectively. Comparisons between group A and group B left ovaries were also showed significant differences of plasma E-cadherin levels (P = 0.003), primordial follicles count (P < 0.001), antral follicles count (P <0.001) in addition to ovarian weight (P <0.001). There were significant differences (P <0.05) of primordial, primary, secondary and antral follicles count between group A and group B both right and left ovaries.

Conclusion E-cadherin plasma level is negatively correlated with animal age and ovarian weight. Higher E-cadherin plasma level associated with higher primordial follicles count. The primordial and primary follicles count was higher in young rabbits while the secondary and antral follicles count was higher in old rabbits.

Keywords E-cadherin, rabbit ovary, ovarian reserve and folliculogenesis

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List of abbreviations: FSH = Follicle stimulating hormone, H&E= Hematoxylin and Eosin, LH = Luteinizing hormone, OR = Ovarian reserve

Introduction

The primary reproductive organs in rabbit females are the ovaries, which produce hormones like estradiol and progestins as well as release mature ova. The ovaries are found at the extremities of the uterine tubes in the abdominal cavity, adjacent to the kidneys.

The rabbit ovaries are so tiny that they are formed as 20×10 mm ovoid formations that are roughly the size of beans. On their surface, growing follicles resemble blister-like structures and range in weight from 0.5 to 0.75 g depending on the activity of the ovarian components [(1)]. A mass of fat surrounds the mesosalpinx region's beginnings as well as the ovaries [(2)].

The ovary is made up of two layers; the outer layer, known as the cortex, houses the oocytes in various developmental stages within their follicles as well as muscle fibers, nerves, and blood vessels, the medulla is the inner layer consist of connective tissue, blood vessels, and nerves. Ovulated ova are nearly certain to enter the funnel because the ostium of the uterine tube, which has fimbriae "finger-like projections" with one of them linked to the anterior end of the ovary, tends to encircle the border of the ovary [(1)]. The rabbit is a species with induced (reflex) ovulation. Rabbits (including ferrets, cats, and camelids) require copulation to cause gonadotropin releasing hormone (GnRH) release from the hypothalamus into the hypophyseal portal system, in contrast to spontaneous ovulatory (humans, dogs, cows, etc.), which have a clearly defined estrous cycle, females used to exhibit estrous behavior every 4-6 days. In order to produce diverse signals in the rabbit females, such as lordosis in the presence of a male or a red or purple vulva color, estrogen operates on the brain. The estrogen release declines when the follicles degrade, and the rabbits go into a non-receptive phase as seen in (figure 1) [(3)].

Figure 1. Ovulation in the rabbit. The ovary of anesthetized rabbit was exposed and observed when the follicle started to ovulate [(4)]

E-cadherin mostly expressed in epithelial cells, it is a single-span transmembrane glycoprotein with five repetitions and a cytoplasmic domain. They play a variety of additional roles as well, including ensuring tissue integrity and resistance to stretching, mechanotransduction, cell signaling, regulation of cell proliferation, apoptosis, survival, and carcinogenesis. They are essential for cell sorting and identification during morphogenesis. Primordial germ cells (PGCs) express E-cadherin, and it also helps PGCs go to the growing gonads where they are encircled by somatic cells that express N-cadherin [5].

Although the majority of the studies on the role of cadherins in gonad development have focused on E- and N-cadherin, and there are only a few studies on VE- and P-cadherins; meanwhile, the global analysis of developing mouse gonad transcriptomes revealed the expression of many other cadherins and protocadherin's [6]. In addition to E-cadherin involvement in the establishment of the germ cell lineage, it participates in oocyte growth, and in the acquisition of meiotic competence during gonad development. Suggesting this protein play a highly relevant role in folliculogenesis [7].

The number of oocytes (primordial follicles) remaining in the ovary is referred to as the ovarian reserve. Female newborns are born with 500,000 to 1 million oocytes; but, over time, follicular atresia and ovulation cause the number of oocytes to slowly decline, leading to menopause. Although ovarian reserve inversely corresponds with age, women of the same chronologic age have significantly different ovarian reserves [8].

Factors that affect the ovarian reserve are oral contraceptives, obesity, smoking, vitamin D status, endometriosis, chemotherapy, and previous ovarian surgery and alcohol usage are several lifestyle factors that have been evaluated for their possible effect on ovarian reserve [9].

Both biochemical testing and ultrasound imaging (biophysical testing) of the ovaries are used in ovarian reserve assessments. Measurements of follicle stimulating hormone (FSH), estradiol (E2), or inhibin B during the early follicular phase, measurements of cycle-day-independent antimullerian hormone (AMH), are other subcategories of biochemical testing of ovarian reserve. The ultrasonographic measures available the ovarian volume measurements and the antral follicle count (AFC), the tests most commonly utilized in clinical practice today [10].

The goal of this study was to assess E-cadherin level as predicting parameter for ovarian reserve.

Methods

Prospective cohort study design, 25 rabbits (*Oryctolagus cuniculus*) were divided into two groups:

1. Group A: young female rabbits (25 sample) aged (10-20 weeks) to:
   - Measuring the plasma levels of ELISA E-cadherin.
   - Histological examination of ovaries for ovarian follicles counting.

2. Group B: old female rabbit (25 sample) aged (>108 weeks) to:
   - Measuring the plasma levels of ELISA E-cadherin.
   - Histological examination of ovaries for ovarian follicles counting.

Each rabbit was weighed with a balance and (prior to euthanasia, blood was collected from the heart). For each group, blood samples were drawn from the heart and placed in a labeled tube, and then the tubes were placed in the centrifuge for (30 minutes) to separate the plasma from the blood well and then euthanized the animals by chloroform that had been soaked in cotton swabs and kept in an airtight chamber for three to five minutes. The animal was then placed on an anatomical stage in the dorsal position and secured to the dissecting table by its four limbs. Then the rabbit was dissected. The ovaries were

extracted from the oviducts and the surrounding tissue, each ovary weighted with sensitive balance. The ovarian tissue was collected and processed for paraffin block and then sectioned and stained. Hematoxylin and Eosin (H&E) stain according to [11] for demonstration of the cellular components of the ovary and follicles at various developmental stages. The histomorphology of the ovarian tissue was examined particularly the number of follicles. Estimation of plasma level of E-cadherin.

Statistical analysis

The data were analyzed using statistical package for social sciences (SPSS) version 23.0 and Microsoft office 2010. The descriptive statistics including mean and standard deviation was measured to describe the data. The groups were compared by applying independent sample t-test (unpaired t-test between 2 continuous variables in different two groups). The degree of association between continuous variables was calculated by Pearson's correlation coefficient (r) and the results were considered statistically significant when p value was equal to or less than 0.05.

Results

The ovaries of both young and old female were yellowish-whitish in due and elongated, triangular in shape, with their bases oriented cranially. Furthermore, these results differed from those of other animal species, such as the African giant rat, whose ovaries were kidney-shaped and pink in color. But like these rats, rabbits also had ovaries that were positioned caudally to the kidneys, with the right ovary being more cranial than the left, and that were suspended from the lumbar muscles by the mesovarium ligament. It was discovered that older rabbits had larger ovaries than younger rabbit. Histologically, the primordial follicle is surrounded by a single layer of flattened granulosa cells the granulosa cells in primordial follicles are tiny, the primary follicle is encircled by granulosa cells that are arranged in a single layer surrounding the oocyte and take on a cuboidal shape in the primary follicle, the secondary follicle is surrounded by a many layers of granulosa cells. A layer known as zona pellucida is secreted between the granulose cells. Significantly increases following antrum development. The cumulus oophorous cells enclosing the oocyte and the granulosa cells lining the follicular wall are divided by the fluid-filled antrum. Typically, all of the oocytes in tertiary follicles are entirely covered by the zona pellucida.

Statistics

Comparisons between group A and group B right ovaries showed significant differences of plasma E-cadherin levels (116.69±33.12 vs. 110.42±34.78; P = 0.003), primordial follicles count (210.0±22.34 vs. 99.40±26.73; P <0.001) and antral follicles count (7.50±2.45 vs. 40.0± 2.89; P <0.001); however there was no significant difference of ovarian weight (0.16±0.11 vs. 0.21±0.13; P = 0.457) for group A and group B right ovaries respectively as presented in table (1).

Comparisons between group A and group B left ovaries were also showed significant differences of plasma E-cadherin levels (P = 0.003), primordial follicles count (P <0.001), antral follicles count (P <0.001) in addition to ovarian weight (P <0.001) as demonstrated in table (2).

Table 1. Comparison of plasma E-cadherin levels, primordial follicles count and ovarian weight between group A and group B right ovaries

Parameters	Group A mean±SD	Group B mean±SD	P value
Plasma E-cadherin (ng/ml)	116.69±33.12	110.42±34.78	0.003 ₸ S
Primordial follicles count	210.0±22.34	99.40±26.73	<0.001 ₸ S
Antral follicles count	7.50±2.45	40.0±2.89	<0.001 ₸ S
Ovarian weight (gm)	0.16±0.11	0.21±0.13	0.457 ₸ NS

₸: Independent sample t test; NS: Not significant (P >0.05); S: Significant (P ≤0.05)

Table 2. Comparison of plasma E-cadherin levels, primordial follicles count and ovarian weight between group A and group B left ovaries

Parameters	Group A mean±SD	Group B mean±SD	P value
Plasma E-cadherin (ng/ml)	116.69±33.12	110.42±34.78	0.003 ₸ S
Primordial follicles count	204.60±26.65	98.90±23.16	< 0.001 ₸ S
Antral follicles count	6.33±2.37	38.30±2.54	< 0.001 ₸ S
Ovarian weight (gm)	0.08±0.05	0.26±0.14	< 0.001 ₸ S

₸: Independent sample t test; NS: Not significant (P >0.05); S: Significant (P ≤0.05)

There were significant differences (P <0.05) of primordial, primary, secondary and antral follicles count between group A and group B both right and left ovaries as demonstrated in (table 3).

Table 3. Comparison of primary, secondary and antral follicles between group A and group B right and left ovaries

Parameters	Group A mean±SD	Group B mean±SD	P value
Primary follicles	95.90±14.27	74.00±12.31	0.004 ₸ S
Secondary follicles	3.29±1.46	92.20±7.88	< 0.001 ₸ S
Left ovary			
Primary follicles	92.80±14.57	74.70±17.67	0.014 ₸S
Secondary follicles	3.71±1.92	87.00±9.72	< 0.001 ₸ S

₸: Independent sample t test; S: Significant (P ≤0.05)

Correlations between plasma E-cadherin level with rabbit's age, ovarian weight and primordial follicles count of group A and group B rabbits were demonstrated in table (4); in group A there was significant positive correlation between primordial follicles count with plasma level of E-cadherin. On the other hand, there were insignificant negative correlation between both rabbits ages and ovarian weights with plasma level of E-

cadherin. While in group B there was insignificant positive correlation between primordial follicles count and plasma level of E-cadherin. Insignificant negative correlation between both rabbits ages and ovarian weights with plasma level of E-cadherin.

Table 4. Correlations between plasma E-Cadherin level with rabbit's age, ovarian weight and primordial follicles count in group A and group B

Parameters		Plasma E-Cadherin	
		Group A	Group B
Age	r	-0.522	-0.135
	p	0.229 NS	0.709 NS
Weight of the ovary	r	-0.534	-0.409
	p	0.173 NS	0.314 NS
Primordial follicles count	r	0.908	0.254
	p	<0.001 S	0.479 NS

Ŧ: Independent sample t test; NS: Not significant (P >0.05); S: Significant (P ≤0.05)

Histologically, the primordial follicle is surrounded by a single layer of flattened granulosa cells the granulosa cells in primordial follicles are tiny. The primary follicle is encircled by granulosa cells that are arranged in a single layer surrounding the oocyte and take on a cuboidal shape in the primary follicle as seen in figures (2 and 3).

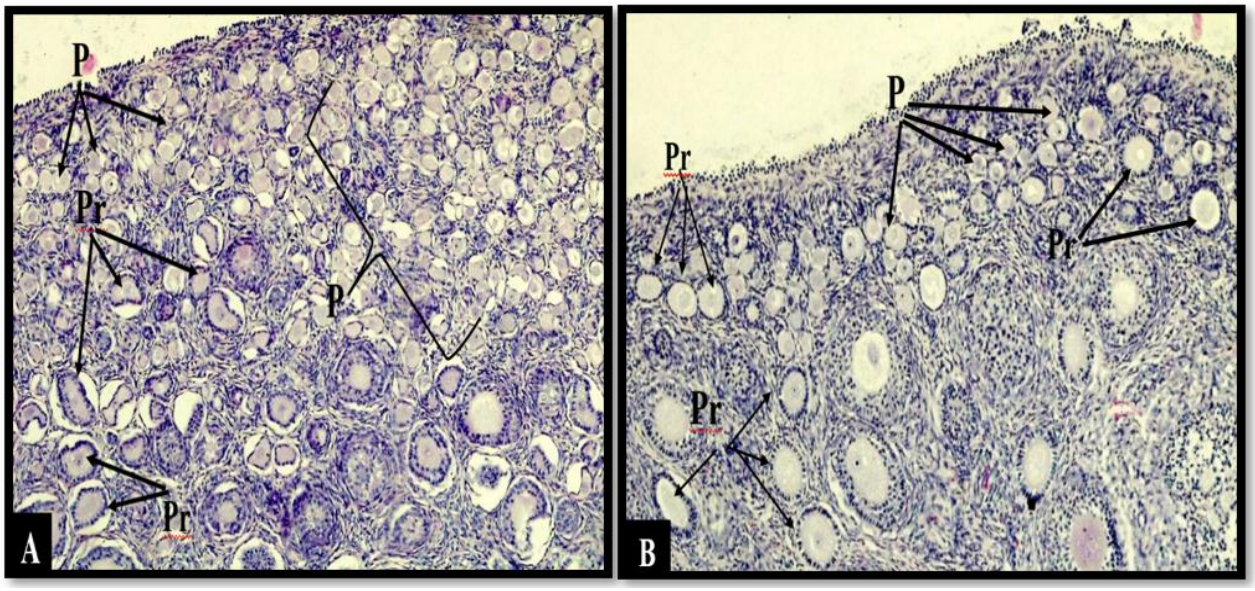

Figure 2. Section of ovarian tissue. in the picture A: in group A showing the (P) primordial follicles, and (Pr) primary follicles, in picture B: in group B showing the flat cells of (P) primordial follicles, and single layer of cuboidal cells in (Pr) primary follicles (10x magnification, H&E)

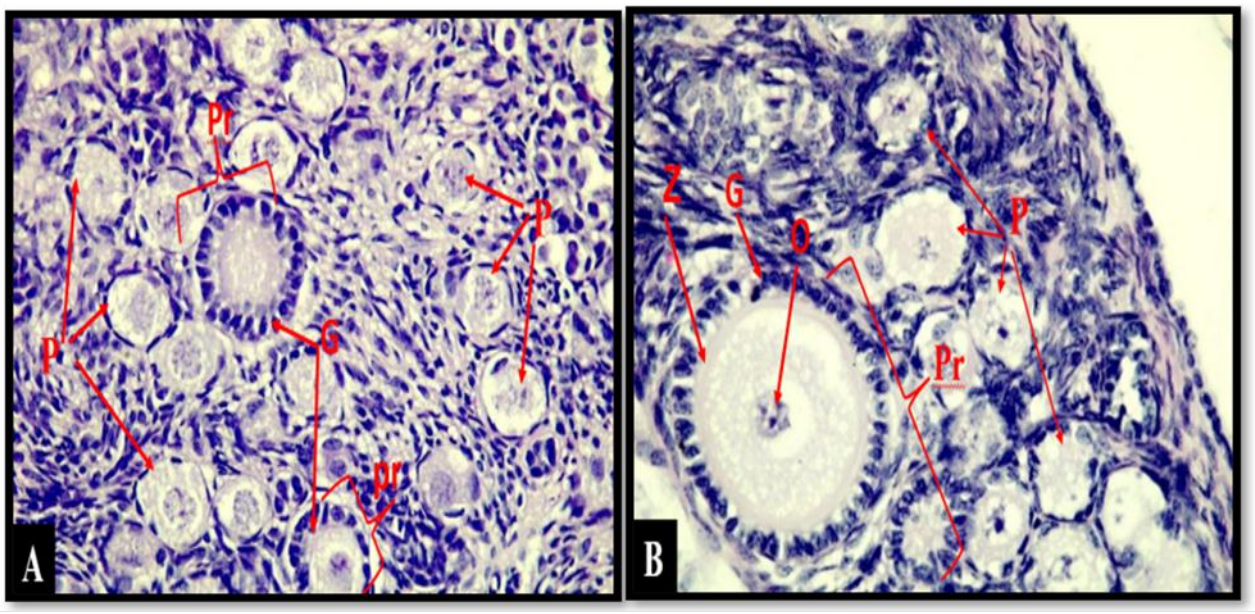

Figure 3. Section of ovarian tissue in the picture A: in group A showing the flat cell in (P) primordial follicle, and single layer of cuboidal cells (G) granulosa cells in (Pr) primary follicle, in picture B: in group B showing the flat cell in (P) primordial follicle, and single layer of cuboidal cells (G) granulosa cells, (Z) zona pellucida and (O) oocyte in (Pr) primary follicle (40x magnification, H&E)

The secondary follicle is surrounded by a many layers of granulosa cells. A layer known as zona pellucida is secreted between the granulose cells as seen in (figure 4 and 5). Significantly increases following antrum development. The cumulus oophorous cells enclosing the oocyte and the granulosa cells lining the follicular wall are divided by the fluid-filled antrum. Typically, all of the oocytes in tertiary follicles are entirely covered by the zona pellucida as seen in figure (6).

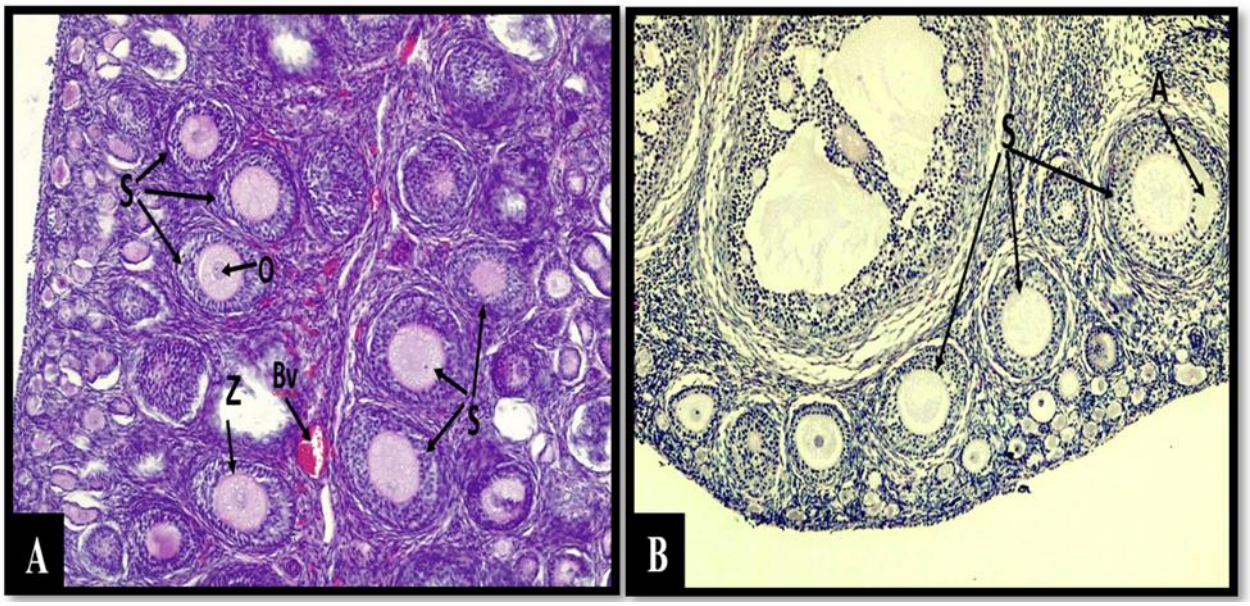

Figure 4. Section of Secondary follicle Showing in picture A: in group A the (S) secondary follicles Surrounded by many layers of granulosa cells, (O) oocyte, (Bv) blood vessel, in picture B: in group B the (S) secondary follicles Surrounded by many layers of granulosa cells, (A) antrum (10x magnification, H&E)

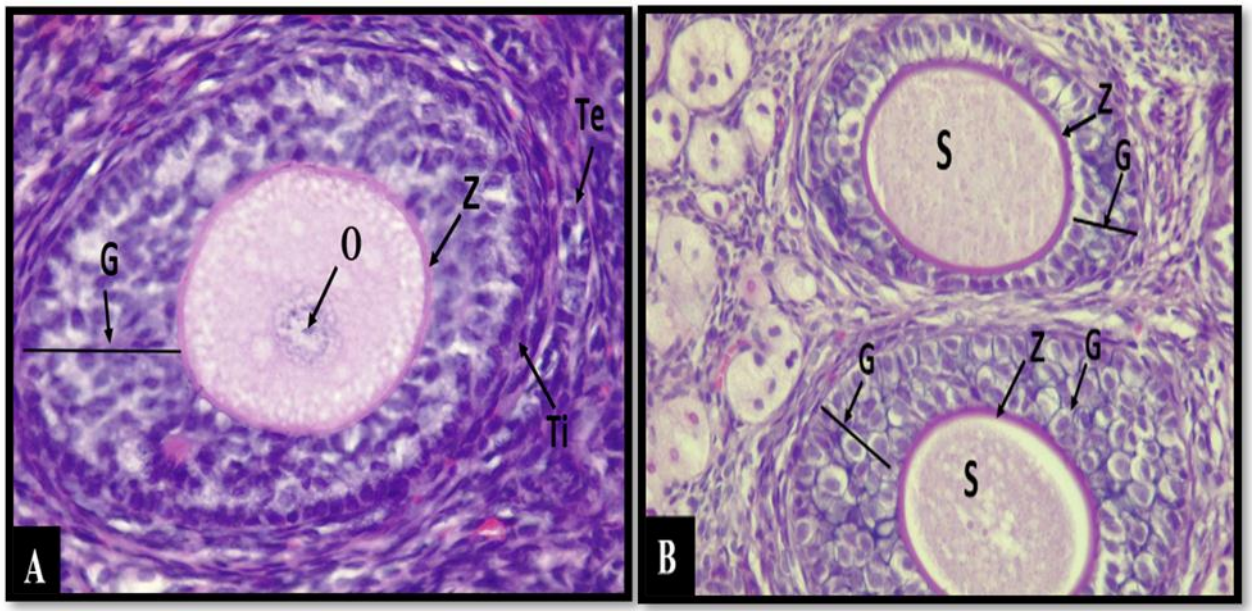

Figure 5. Section of Secondary follicle in picture A: in group A Surrounded by (Te) theca externa, (Ti) theca interna, many layers of (G) granulosa cells surrounding the (Z) zona pellucida and (O) oocyte, in picture B: in group B Showing the (S) secondary follicles Surrounded by many layers of (G) granulosa cells, (Z) zona pellucida (40x magnification, H&E)

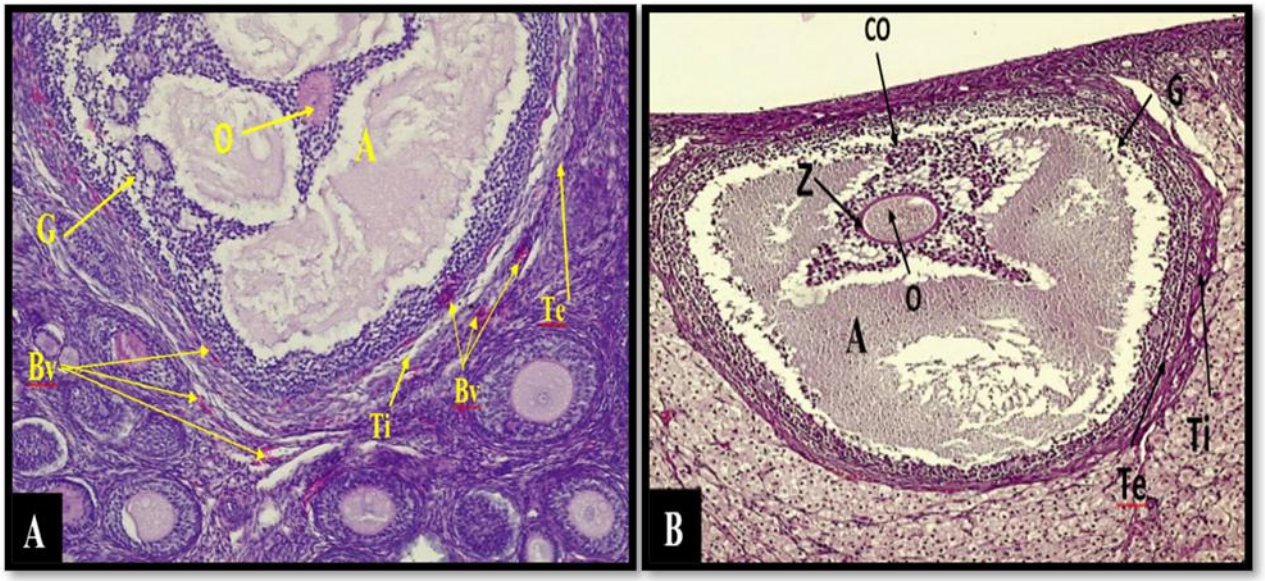

Figure 6. Section of graafian follicle in picture A: in group A Showing the (Te) theca externa, (Ti) theca interna, (G) granulosa cells surround the (A) antrum and (O) oocyte, (Bv) blood vessel, in picture B: in group B showing the (GF) graafian follicle consist of (Te) theca externa, (Ti) theca interna, (CO) cumulus oophorous, (G) granulosa cells surround the (A) antrum and (O) oocyte (10x magnification, H&E)

Discussion

Folliculogenesis occurs within the cortex of the ovary. The follicles in the cortex were present in a wide range of different size representing various stages of folliculogenesis. The ovary of rabbit consisted of narrow cortex externally and medulla internally, it was covered by pseudocolumner at group A (young rabbits) and then became more cuboidal at group B (old rabbits), the whole organ surrounded by connective tissue tunica. These findings were supported by findings of other studies that mentioned the medulla to be vascularized and contain more stroma (smooth muscle fibers and fibroblast). The ovarian follicles were mostly located at the cortex while the blood vessels were located in the medulla of the ovary [12-14].

Morphologically speaking, the ovaries of the younger rabbit (group A) were found to be smaller in size, lighter in weight than that of older rabbit (group B) and the animal weight as well. These findings, could be due to the still developing ovaries in young group (group A) and more adipose tissue deposition and fibrous tissue accumulation in those ovaries of older animal (group B) due to multiple ovulations had been happened throughout the life of these old animals rendering the ovaries less reproductive decrement in number of follicles and increment of ovarian (fibro-adipose) stroma that increase the ovary weight. The finding of the current study agreed with Parisi et al. that found a linear relationship between body weight and age of premenarchal girls and a significant curvilinear relationship of the relative ovarian weight with age [15]. In the current study the ovary weight was increasing with advance of age in rabbit from mean ovarian weight (0.16±0.11) in group A to mean of (0.21±0.13) in group B. Pavlik, et al. who stated and disagreed by no relation between ovarian volume and body weight and there was a significant decrease in ovarian volume with decade of age [16].

In the current study, many features were found in favor of the right ovary, these findings suggest that the right ovary is dominant. The right ovary in this study was found to have more number of follicles specifically the

primordial and primary types in right more than left ovary in both group A and B as seen in figures (2 and 3) in the experiment, this may give the right ovary more chance to develop and produce the dominant follicle of the follicular pool in the ovary, this dominant follicles, the one that eventually become a mature egg and ovulated is more likely produced by right ovary, this suggestion is adopted by the study published in the European Society of Human Reproduction and Embryology demonstrated that for both fertile and infertile women 55% of all ovulation came from the right ovary. These findings of right ovary to have a larger number of primordial and primary follicles may be due to many physiological and anatomical differences between the right and left ovaries, one of them may be the venous drainage for each ovary is different [(17)].

The secondary follicles were seen evidently in the ovarian sections prepared in this current study by their unique features, when the primary oocyte was located centrally within the secondary follicle and surrounded by multilayered (3-4 cells) thick, granulosa cells that secret a glycoprotein to surround the primary oocyte by a layer called zona pellucida that appeared stained pink (acidophilic). External to the zona pellucida and multilayered granulosa cells, there was vascular layer of theca interna which is a steroid-secreting layer and external to the theca interna, the fibrous layer theca externa as seen in figures (4 and 5). These findings were described by other studies [(12,18)]. In the current study it was found that the number of secondary follicles was less in group A (younger) than group B (older).

The graafian follicles seen in this study was like a three-dimensional structure with a central antrum surrounded by about six types of histologic components, namely, theca externa more external, theca interna vascular and steroid hormone secreting. basal lamina, granulosa cell layer, oocyte (secondary) and follicular fluid as described in figure (6), these findings were convenient with previous researchers and their scientific works [(19)].

This type of follicles can be defined structurally by presence of antrum or cavity that contains follicular fluid, so sometimes we can call them as antral follicle [(19)]. Theca externa layer contained (antral) smooth muscle fibers or cells that might contract during ovulation which could trigger the ovulation process. Theca interna was composed of differentiated cells that are capable of hormone secretion and regulation like androgen hormone, FSH and luteinizing hormone (LH) [(20)].

The biostatistics of the current study was very helpful tool to elaborate meaningfully about the different parameter measured in this work in order to postulate the concept beyond the differences of values measured correspondingly i.e., when study the table (1) and (2) that compare group A (younger) and group B (older), in term of plasma level of E-cadherin and the other parameters mentioned in these tables sequentially, we found that group A is significantly showed higher plasma level of E-cadherin when compared to that of group B, this would give the clue that the younger aged animals or in other words, the ovaries in early reproductive life or development showed higher E-cadherin level, which supports its fundamental role of in promoting the ovarian sexual functions and differentiating of its cellular components. If one compares these findings higher plasma level of E-cadherin with significantly higher primordial follicles counts versus the group B, this would support the same conclusion of [(21)] that E-cadherin or sometimes called cadherin-1 would sustain the dominant primordial follicles pool [(22)].

In the current study, the follicular counting was a tedious work by examining the slides in each group A and B that slides should be seen by different magnification power 4x, 10x, 40x respectively. There was a highly significant differences between primordial follicle and primary follicle count in group A (younger) > than group B (older), on the contrary the secondary and antral follicles numbers were more in group B > than group A with a highly significant p value. This finding might establish the following conclusive statement, that the younger the ovarian tissue, the higher the primordial follicle and primary follicle count, while in older animal with > 108 weeks age and

more reproductive activity, one can see less number of the primordial follicle due to maturation them (exhaustion of primordial follicle frequent ovulation), So the young, ovarian tissue also associated with a higher plasma level of E-cadherin and sustain more primordial follicles reserve.

The number of ovarian follicles were counted and differentiated according to type and chronological age of ovarian tissue (from young to old animals). It was found that there was a depletion of the number of primordial follicles with chronological age of the animal model (where the total primordial follicle count was (210) and AFC was (7.50) in group A, while in group B the primordial follicle count (99.4) and antral follicle count AFC (40) this finding was agreed by many previous researches on human and animal model [23].

Many of non-growing follicle (NGF) (resting primordial follicles) were triggered by special mechanism to enter into growth phase and as a result there will be decline in the total number of ovarian reserves with increasing age. This conclusion is studied and stated in human by Park, et al. (2021) who proved with his colleagues that the number of oocytes eventually comprise a pool of PFs that decline in number throughout reproductive female life [24]. This suggestion is the first time to be studied thoroughly by this current work and no previous study had suggested such conclusion, despite many factors that were identified as a predictor marker for ovarian reserve like AMH, FSH, Inhibin B [25].

This study concluded:

1. Higher plasma E-cadherin was associated with higher primordial follicles number.
2. The younger the reproductive age of the animal, the higher the plasma level of E-cadherin protein.
3. The primordial and primary follicles count was higher in young rabbits while the secondary and antral follicles count was higher in old rabbits.
4. The ovarian reserve (primordial follicle pool) was significantly correlated with plasma level of E-cadherin, which can suggest the E-cadherin as predictor marker for primordial follicle ovarian reserve (OR).
5. Ovarian weight and volume were increased with advancing age of rabbit.

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Author contribution

Both authors contributed in conception, study design, acquisition of data, manuscript writing and final approval of the version to be published.

Conflict of interest

There is no conflict of interest.

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