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E-CADHERIN PLASMA LEVEL AND IMMUNOHISTOCHEMICAL EXPRESSION FOR ASSESSMENT OF OVARIAN RESERVE IN RABBIT

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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 ovaries are found at the extremities of the uterine tubes in the abdominal cavity, adjacent to the kidneys. The rabbit ovaries are so tiny. 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 of the consist of connective tissue, blood vessels, and nerves. E-cadherin is a single-span transmembrane glycoprotein with five repetitions and a cytoplasmic domain. It plays a variety of roles as well, including ensuring tissue integrity and resistance to stretching, cell signaling, regulation of cell proliferation, apoptosis, survival, and carcinogenesis.

Aims of this study: Assessment of E-cadherin level and immunohistochemical expression as predicting parameter for ovarian reserve.

Material and methods: female's rabbits (Oryctolagus cuniculus) of age (10 to more than108 weeks), weighting 600-3000 gm 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 and then euthanized the animals. Specimens of ovaries were placed on positively charged slides of immunohistochemical staining.

Results: The current study showed significant differences of plasma E-cadherin levels ($p=0.003$), immune-histochemistry expression ($p=0.024$), 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 ($p=0.003$), immune-histochemistry expression ($p=0.01$) primordial follicles count ($p < 0.001$), antral follicles count ($p < 0.001$) in addition to ovarian weight ($p < 0.001$).

Conclusions: E-cadherin expression (IHC) and plasma level are negatively correlated with animal age and ovarian weight. Higher E-cadherin (plasma level & IHC) associated with higher primordial follicles count. E-cadherin expression positive correlation with plasma level of E-cadherin.

Key words: E-cadherin, ovarian reserve, rabbit ovary and primordial follicles.

Introduction:

Ovaries of rabbit:

The primary reproductive organs in 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 [19].

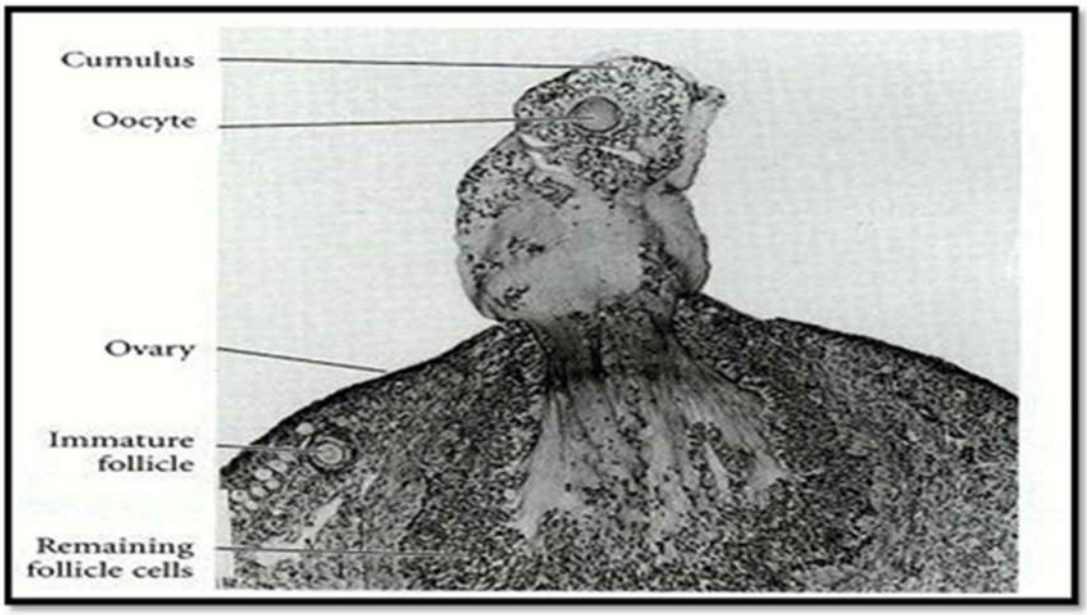

Figure (1) Ovulation in the rabbit. The ovary of a living, anesthetized rabbit was exposed and observed. When the follicle started to ovulate, the ovary was removed, fixed, and stained.

E-cadherin:

Mostly expressed in epithelial cells, E-cadherin 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 [3].

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 [4]. 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 [5].

What is ovarian reserve:

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 [6].

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 [7].

Both biochemical testing and ultrasound imaging (biophysical testing) of the ovaries are used in ovarian reserve assessments. Measurements of 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 [8].

Materials and Methods:

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

1- Group A: young female rabbits (25 sample) aged (10-20 w/c) to:

- Measuring the plasma levels of Elisa E-cadherins.
- Histological examination of ovaries for ovarian follicles counting.
- Immunohistochemical examination of ovaries for assessment of E-cadherin expression.

2- Group B: old female rabbit (25 sample) aged (>108 w/c) to:

- Measuring the plasma levels of Elisa E-cadherins.

- Histological examination of ovaries for ovarian follicles counting.
- Immunohistochemical examination of ovaries for assessment of E-cadherin expression.

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 stain according to [9] 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. Immunohistochemical E-cadherin polyclonal antibody according to [10] to demonstrate follicular tissue reactivity, then the immunohistochemical expression of the E-cadherin marker was evaluated in rabbit ovaries and 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 errors were 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:

Immunohistochemistry of Ovarian Tissue:

The Aperio algorithms software program was utilized to perform a quantitative assessment of the intensity of reactivity for E-cadherins antibody. General distribution of E-cadherin immunohistochemical reactivity in the ovary was more in the medulla rather than the cortex except the outer tunica albuginea of ovary. In medulla, the E-cadherin was localized more in showed interstitial tissues but it is more expressed in the follicular cellular layer while it was highly presented in the cytomembrane of the oocyte more than the follicular cell layer. Showed very high expression of E-cadherin in zona pellucida but less in granulosa cells rather than zona pellucida.

Statistics:

Comparisons between group A and group B right ovaries showed significant differences of plasma E-cadherin levels (116.69 ± 4.61 vs. 110.42 ± 2.84; p=0.003), immunohistochemical expression (7717 ± 459 vs. 7025 ± 256; p=0.024), primordial follicles count (210.0 ± 18.66 vs. 99.40 ± 3.35; p< 0.001) and antral follicles count (7.50 ± 1.48 vs. 40.0 ± 1.57; p<0.001); however there was no significant difference of ovarian weight (0.16 ± 0.06 vs. 0.21 ± 0.02; p= 0.457) between group A and group B right ovaries as presented in (table 1).

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

Parameters	Group A	Group B	*p* value
Plasma E-cadherin (ng/ml)	116.69 ± 4.61	110.42 ± 2.84	0.003 Ŧ S
IHC expression	7717 ± 459	7025 ± 256	0.024 Ŧ S
Primordial follicles count	210.0 ± 18.66	99.40 ± 3.35	< 0.001 Ŧ S
Antral follicles count	7.50 ± 1.48	40.0 ± 1.57	< 0.001 Ŧ S
Ovarian weight (gm)	0.16 ± 0.06	0.21 ± 0.02	0.457 Ŧ NS

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

Comparisons between group A and group B left ovaries were also showed significant differences of plasma E-cadherin levels (p=0.003), immunohistochemical expression (p=0.01) 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 2: Comparison of plasma E-cadherin levels, immunohistochemical expression, primordial follicles count and ovarian weight between group A and group B left ovaries

Parameters	Group A	Group B	*p* value
Plasma E-cadherin (ng/ml)	116.69 ± 4.62	110.42 ± 2.84	0.003 Ŧ S
IHC expression	7317 ± 446	6883 ± 172	0.010 Ŧ S
Primordial follicles count	204.60 ± 15.68	98.90 ± 4.24	< 0.001 Ŧ S
Antral follicles count	6.33 ± 1.41	38.30 ± 1.50	< 0.001 Ŧ S
Ovarian weight (gm)	0.08 ± 0.01	0.26 ± 0.02	< 0.001 Ŧ S

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

Correlations between plasma E-cadherin and E-cadherin IHC expression with rabbit's age, ovarian weight and primordial follicles count of group A rabbits were demonstrated in table 3;

Table 3: Correlations between IHC and plasma E-Cadherin levels with rabbit's age, ovarian weight and primordial follicles count of group A

Parameters		IHC expression	Plasma E-Cadherin
Age	r	-0.277	-0.522
	p	0.547 NS	0.229 NS
Weight of the ovary	r	-0.519	-0.534
	p	0.188 NS	0.173 NS
Primordial follicles count	r	0.887	0.908
	p	0.001 S	< 0.001 S

r: Pearson's correlation coefficient; NS: Not significant ($p > 0.05$); S: Significant ($p \leq 0.05$).

Correlations between plasma E-cadherin and IHC expression with rabbit's age, ovarian weight and primordial follicles count in group B rabbits, as demonstrated in (table 4) there was:

Table 4: Correlations between IHC expression and plasma E-Cadherin with rabbit's age, ovarian weight and number of primordial follicles of group B

Parameters		IHC expression	Plasma E-Cadherin
Age	r	-0.537	-0.135
	p	0.110 NS	0.709 NS
Weight of the ovary	r	-0.795	-0.409
	p	0.018 S	0.314 NS
Numbers of primordial follicles	r	0.795	0.254
	p	0.006 S	0.479 NS

r: Pearson's correlation coefficient; NS: Not significant ($p > 0.05$); S: Significant ($p \leq 0.05$).

Correlations between plasma E-cadherin and E-cadherin IHC expression in both A and B group rabbits: There was significant positive correlation between plasma and immune-histochemistry E-cadherin expression of group A rabbits ($r=0.960$ & $p < 0.001$); although there was insignificant positive correlation between plasma level and immune-histochemistry E-cadherin expression of group B rabbits ($r=0.456$ & $p=0.118$) as presented in (table 5).

Table 5: Correlations between plasma and IHC E-Cadherin in group A and group B rabbit's

Group	Pearson's correlation coefficient (r)	*p* value
Group A	0.960	< 0.001 S
Group B	0.456	0.118 NS

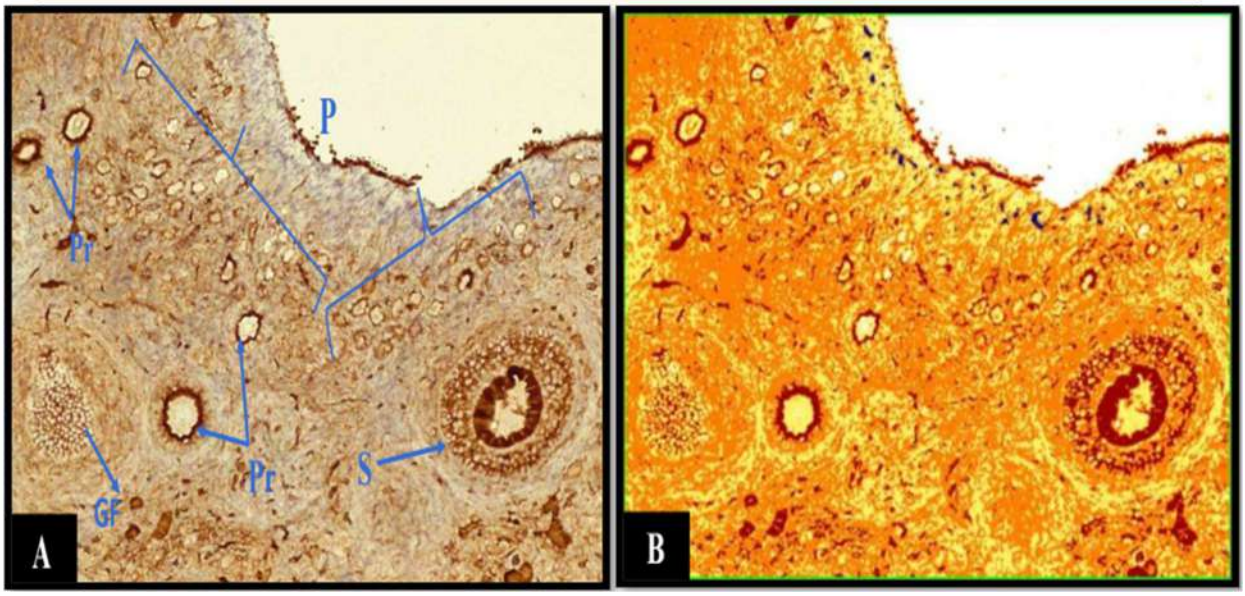

Figure (2) Section of the ovarian tissue in group A showing: A: The expression E-cadherin antibody reactivity in (P) primordial follicles, (Pr) primary follicles, (S) secondary follicle and (GF) graafian follicle (10x magnification, IHC), B: Shows annotation E-cadherins reactivity by Aperio in the primordial, primary, and secondary follicles represented by positivity color ranging between orange to red.

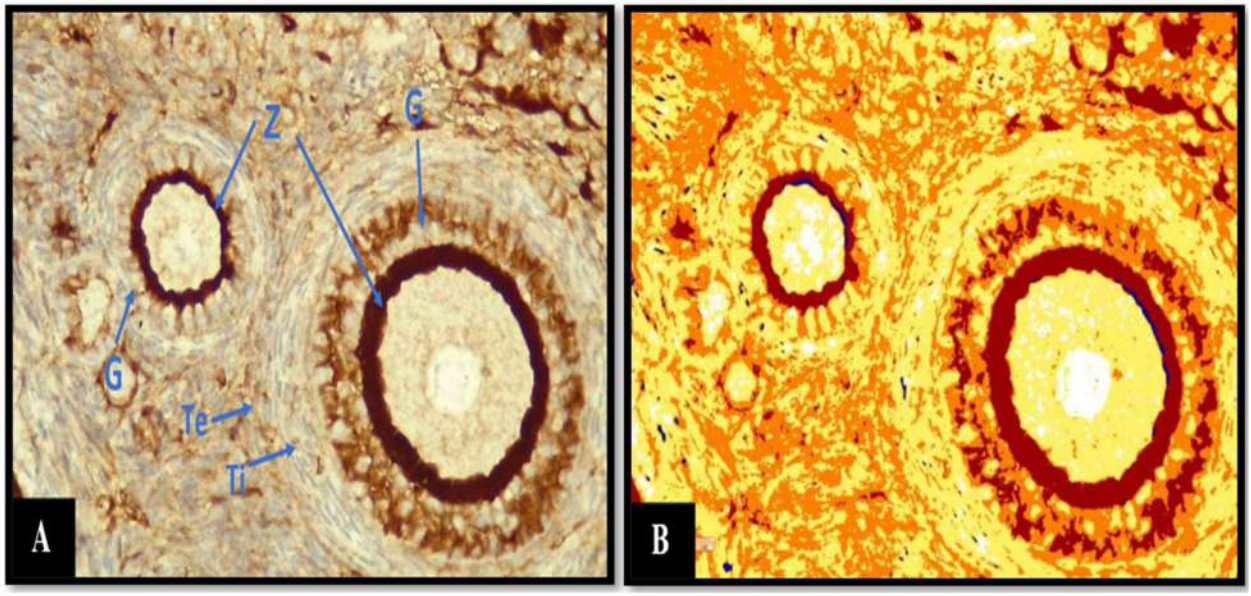

Figure (3) Section of ovarian tissue shows in group A; A: The expression E-cadherin antibody reactivity in (S) secondary follicle, (Te) theca externa, (Ti) theca interna, (G) granulosa cells and (Z) zona pellucida (10x magnification, IHC), B: Shows annotation of E-cadherins reactivity by Aperio in the secondary follicle represented by positivity color ranging between orange to red.

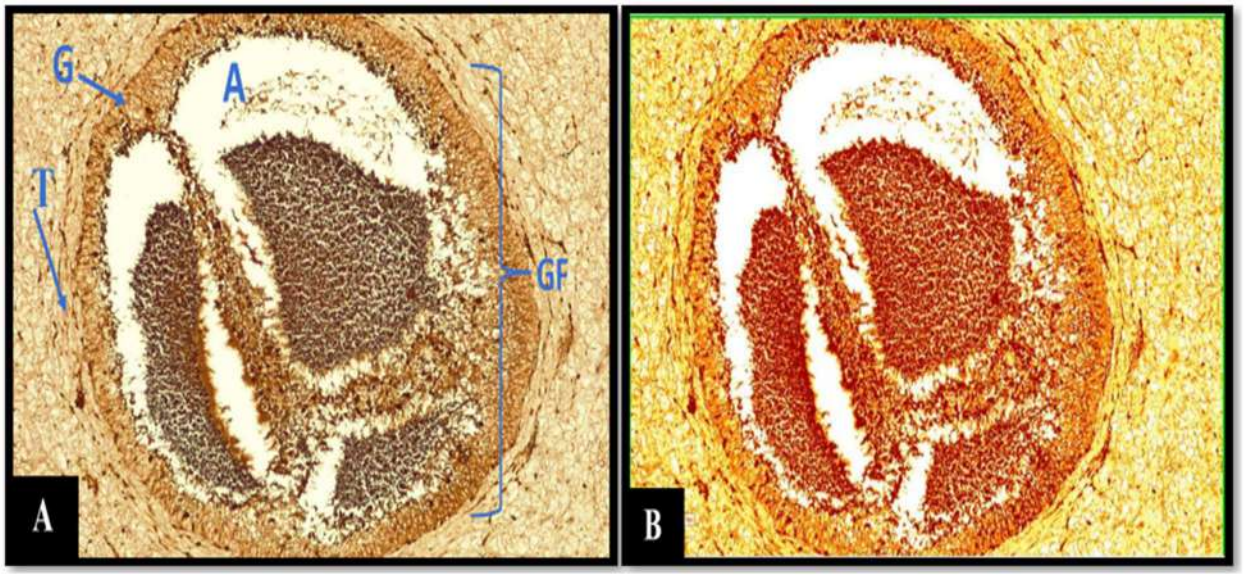

Figure (4) Section of ovarian tissue in group A; A: The expression E-cadherin antibody reactivity in (GF) graafian follicle, (T) theca layer, (G) granulosa cells, (A) antrum (10x magnification, IHC), B: Shows annotation of E-cadherins reactivity by Aperio in the graafian follicle represented by positivity color ranging between orange to red.

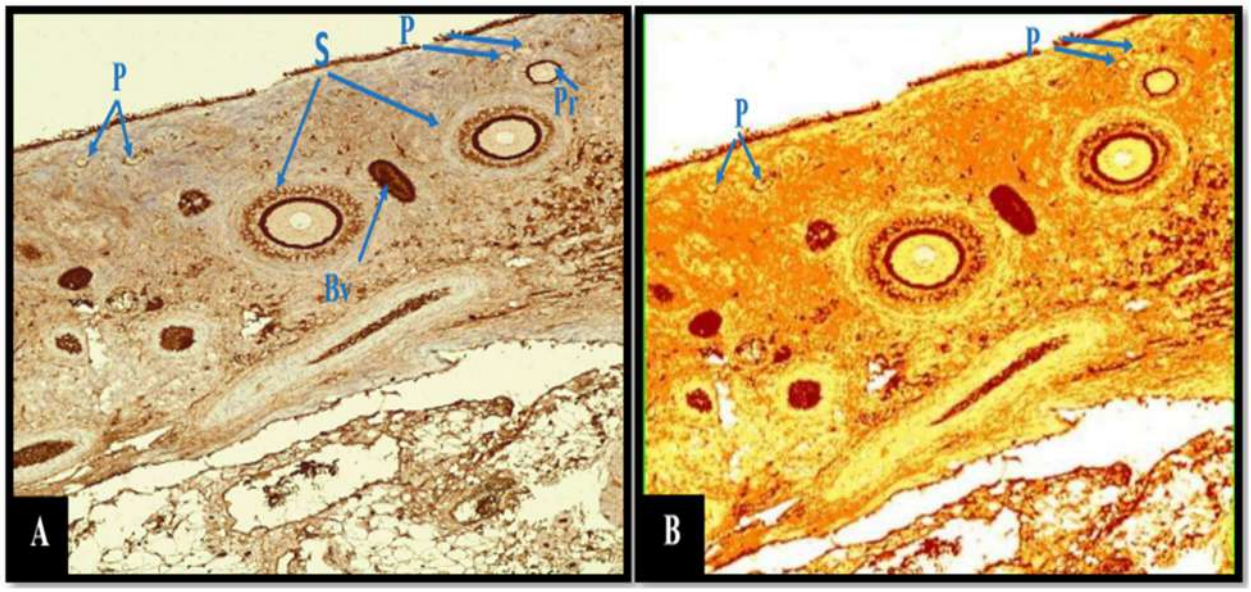

Figure (5) Section of ovarian tissue in group B Showing: A: The expression E-cadherin antibody reactivity in (P) primordial follicles, (Pr) primary follicle, (S) secondary follicle, (Bv) blood vessels (10x magnification, IHC). B: Shows annotation E-cadherins reactivity by Aperio in follicles represented by positivity color ranging between orange to red.

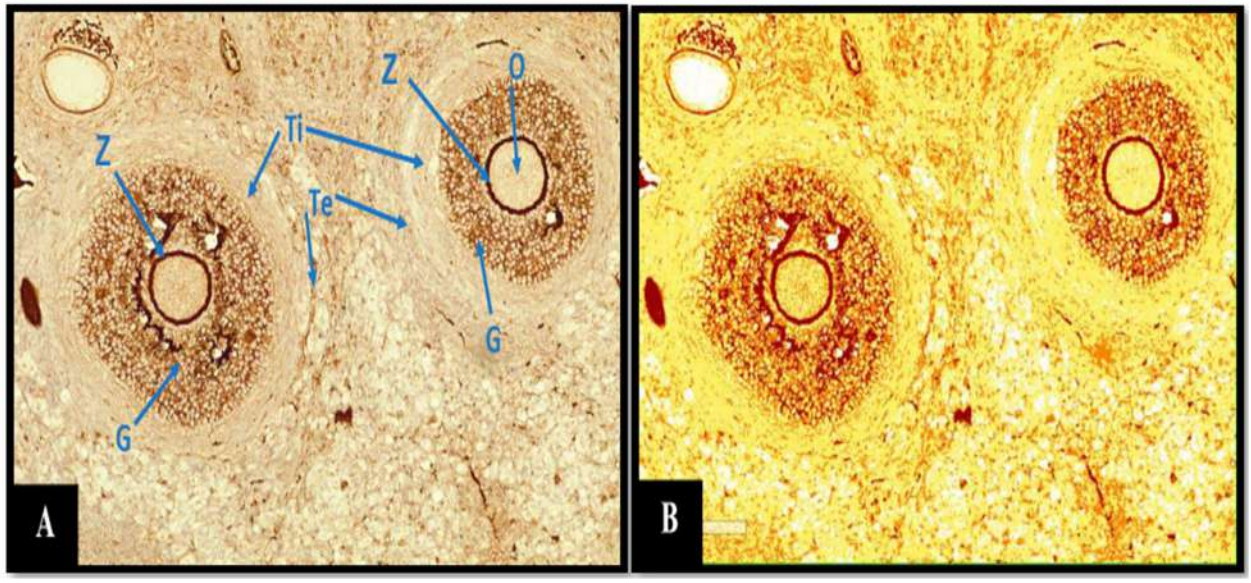

Figure (6) Section of secondary follicle in group B Showing: A: The expression E-cadherin antibody reactivity in (S) secondary follicles showing (Te) theca externa, (Ti) theca interna (G) granulosa cells, (Z) zona pellucida and (O) oocyte (10x magnification, IHC), B: Shows annotation E-cadherins reactivity by Aperio in the secondary follicle represented by positivity color ranging between orange to red.

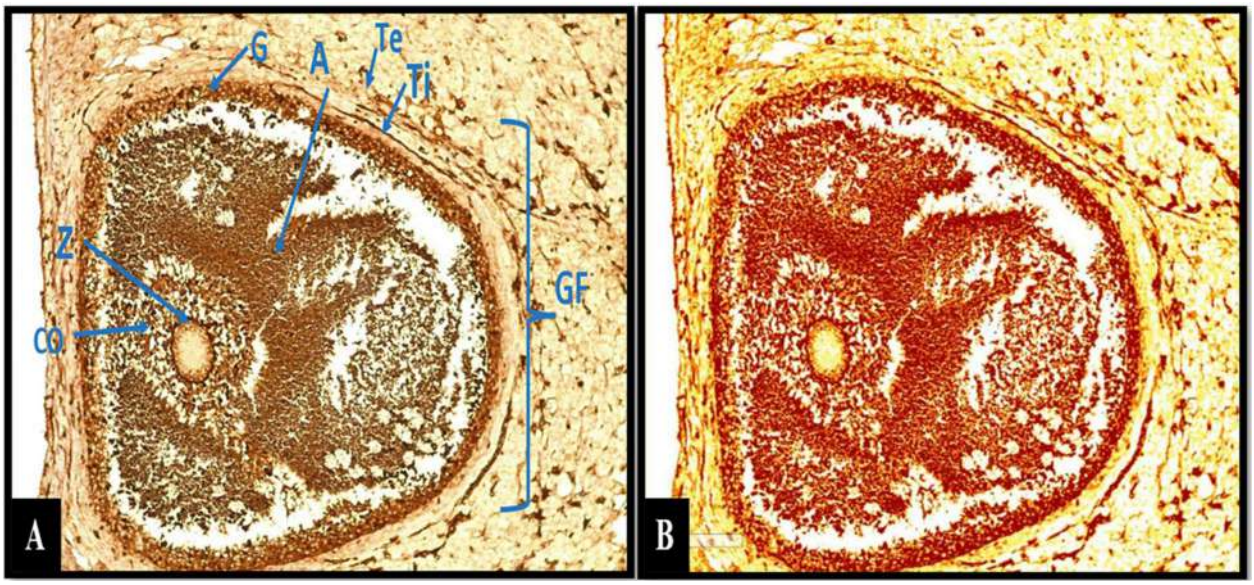

Figure (7) Section of ovarian tissue shows in group B showing: A: The expression E-cadherin antibody reactivity in (GF) graafian follicle, (Te) theca externa, (Ti) theca interna, (G) granulosa cells, (A) antrum, (CO) cumulus oophorous and (Z) zona pellucida (10x magnification, IHC). B: Shows annotation of E-cadherins reactivity by Aperio in the graafian follicle represented by positivity color ranging between orange to red

Discussion:

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 with decrement in number of follicles and increment of ovarian (fibro-adipose) stroma that increase the ovary weight. The finding of the current study as agreed by many other authors' work like parisi.N, that started the linear relationship between body weight , age and a significant curvilinear relationship of the relative ovarian weight with age, although it an article dated back to on dog since there is no newly documental work evaluating the age changes of ovary weight in rabbit [20]. The ovary weight was increasing with advance of age in rabbit from mean ovarian weight (0.16 ± 0.06) in group A to mean of (0.21 ± 0.02) in group B. Pavilik, et al 2000 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 [21].

Immunohistochemical expression in group A:

The positive E-cadherin immunohistochemical reacting has been evaluated and measured quantitively by the Aperio software application which is used to be caused by the researcher. The E-cadherin reactivity was ranging between light brown to dark brown color.

According to the results observed in the current work it was clearly seen that the E-cadherin was

differently expressed according to different cells layer of the follicles or according to the group of experiment. Then, the stage of development of different types of follicles and even the age of organism plays a role to determine the intensity and the site of maximum E-cadherin expression in ovarian tissue which ultimately will give an idea about a site availability of E-cadherin expression. Many studies had stated that the crucial role of E-cadherin in ovarian functional aspect was in maintaining primordial follicles pool (PF pool), although the underlying molecular mechanism is still unexpressed, knowing that the E-cadherin protein is localized particularly in cellular membrane of the oocyte in PF.

In the current study, it was clearly seen that the E-cadherin expression was localized mainly around the oocyte mainly in the granulosa layer which is responsible for the main production cells of E-cadherin while in primary follicles the maximum E-cadherin expression was in zona pellucida layer and in granulosa cell layer as seen (figure 2), and in the secondary follicle the expression was again in the granulosa cells and zona pellucida as seen in (figure 3), these findings of E-cadherin localization would support the hypothesis of the significant role of E-cadherin in cellular integrity and promotion of further development of primordial follicles in to other stages folliculogenesis. As conclusion, E-cadherin play a crucial role in the maintenance of PF pool by promoting and supporting the follicular structural stability that had a pig role in sustaining the female reproduction and fecundity [11],[12].

In graafian follicles, the E-cadherin was expressed in all layers of antral follicles more specifically in granulosa cell rather than other layers although this was not the exact story mentioned by [14], who found that E-cadherin reactivity was highly detected and expressed in all layers of follicular cell as seen in (figure 4) [13]

Immunohistochemical expression in group B:

The previous section was concerned about the description and analysis of the result for group A. While in group B, the story of immunohistochemical expression of E-cadherin was almost similar when the E-cadherin reactivity was localized in cytomembrane of oocyte in follicular cell layer and highly expressed in zona pellucida as seen in (figures 5,6,7) , the only differences observed was that the reactivity of E-cadherin was less in group B which might indicate the decreasing availability of E-cadherin protein with increasing the age, and more availability in early developmental stages of ovaries in younger animals. This explanation was supported by the work of Piprek who stated the crucial role of E-cadherin in many aspects of sexual development, gametogenesis, gonad development, functions and fertilization [13].

Statistics:

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 comparison group A (younger) and group B (older), in term of plasma level of E-cadherin and the other parameters mentioned in this table sequentially, we found that group A is significantly showed higher plasma level of E-cadherin and more immunohistochemical expression if 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 and expression which support the fundamental role of this important protein in promoting the ovarian sexual functions and differentiating of its cellular components if one compares these findings of higher plasma level and expression of E-cadherin with significantly higher primordial follicles count in group A versus the group B (210 in group A > 99.40 in group B; $p< 0.001$) this would support the same conclusion of [15] that E-cadherin or sometimes called cadherin-1 would sustain the dominant primordial follicles pool (PFs) [11].

The current study showed that oocyte derived cadherin-1 performed a vital function in sustaining PFs in rabbit ovaries, as well as, establishing the cytoskeleton of PFs structure by promoting cell-cell adhesion between oocyte and surrounding pregranulosa cells. The same idea was observed in left ovary which clue for the bilateral symmetry of reproductive development and function.

In the current study, the follicular counting was a tedious work by examining the slides in each group A and B these slides should seen by different magnificent power 4x, 10x, 40x respectively. 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 antral follicle count 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 [16].

Many of nongrowing 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, S.U who proved with his colleagues that the number of oocytes eventually comprise a pool of PFs that decline in number throughout reproductive female life [17]. This suggestion is the first time to be studied thoroughly by this current work and no previous study suggest such conclusion, despite many factors are identified as a predictor marker for ovarian reserve like AMH, FSH, Inhibin B [18].

To the best of our knowledge, there was no previous work had depended new biomarker like E-cadherin as a predictor for ovarian reserve or even as indicator for the reproductive and functional aspect neither in human subjects nor in animal models. Thus, one can adapt this conclusion of the importance of E-cadherin estimation for such role.

Conclusion:

1. E-cadherin sustain the large number of primordial follicles. Higher E-cadherin (plasma level & IHC) associated with higher primordial follicles.
2. The younger the reproductive age of the animal, the higher the plasma level of E-cadherin protein.
3. E-cadherin immunohistochemical expression and plasma level are negatively correlated with organism age and ovarian weight.

4. E-cadherin IHC expression positively correlated with plasma level of E-cadherin.
5. The ovarian reserve (primordial follicle pool) was significantly correlated with plasma level of E-cadherin and IHC reactivity which can suggest the E-cadherin as predictor marker for ovarian PF (OR).
6. There was a significant positive correlation between the plasma level of E-cadherin and its IHC reactivity in ovarian tissue the highest E-cadherin IHC reactivity was in zona pellucida.
7. Ovarian weight and volume were increased with advancing age of rabbit.
8. E-cadherin IHC reactivity was higher in younger rabbit than older.
9. Primordial follicle pool number (OR) is higher in younger > older animals.

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