Vet.Med.J.,Giza. Vol.50, No.4. (2002):875-894.

RELATIONSHIP OF BIOCHEMICAL CONSTITUTES OF FOLLICULAR FLUID, HISTOPATHOLOGICAL CHARACTERISTICS OF OVARIES AND IN VITRO MATURATION OF OOCYTES IN BUFFALO COWS DURING DIFFERENT STAGES OF ESTROUS CYCLE

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Received: 20. 8. 2002

Accepted: 2. 10. 2002

SUMMARY

Forty six of buffalo ovaries were collected from slaughter houses. Predicting stages of estrous cycle were identifiable according to the cyclic changes of the corpus luteum (Stage I, day 1-4; stage II, day 5-10; stage III, day 11-17; stage IV, day 18-21of the estrous cycle). Oocytes recovered from different stages were classified according to their, as good quality (compact and partially compact cumulus), and poor quality (expanded, denuded and degenerated) oocytes. Good quality oocytes were in-vitro cultured to study the influence of different stages of estrous cycle on in-vitro maturation of oocytes. Histopathological studies of right and left ovaries from different stages were performed to investigate the growth and atresia of follicles that aid in understanding the complexity of events associated with maturation of buffalo follicle and oocytes. Moreover, follicular fluids were collected and analyzed for intra-follicular hormonal and biochemical constituents.

Stage III provided the highest rate of good quality oocytes that resumed meiosis to metaphase II. Histopathological findings revealed atrestic follicles and degeneration of granulose cells at different stages of the estrous cycle. Follicular progesterone and estradiol levels and their ratio (P: E) were significantly peaked ($P < 0.01$) during stage III (day 11-17), while testosterone started to increase at stage IV and reached its maximum at stage I of the cycle. Total protein, albumin, globulin, triglycerides, sodium, potassium, calcium and inorganic phosphorus were significantly increased ($P < 0.01$) in follicular fluid during stage III. On the other hand, the lowest levels of total lipids, cholesterol, creatinine, urea and transaminase enzyme (AST & ALT) were observed at stage III.

INTRODUCTION

Ovaries obtained from the abattoir constitute an economical source of oocytes, although the quality of these oocytes is highly variable (Brackett and Zuelke, 1993). The use of defined morphological criteria in the selection of cumulus-oocyte complex has led to limited improvement in the identification of the oocytes that well succeed in in-vitro maturation and fertilization in cattle (De-Loos et al., 1989). It is possible that the intrafollicular environment to which oocytes are exposed is a major cause of the variability in development competence of the oocytes (Callesen et al., 1986).

Hormonal and biochemical constituents of follicular fluid fluctuate considerally with the stage of the estrous cycle, follicle size and follicle status (Kruip and Dielman, 1985). These changes in follicular fluid may influence steroidogenesis, oocyte maturation, ovulation and transport of the oocyte to the oviduct as well as preparation of the follicle for subsequent corpus luteum formation and function (Peters and McNatty, 1980). It is not known how the biochemical constituents of the follicular fluid interact with the gamete cell and surrounding supportive cells. To understand the events which influence oocyte maturation, it requires chemical examination of the follicular fluid during different stages of estrous cycle. Less is known about the effect of stage of cycle on, chemistry of the follicular fluid, oocyte quality and maturation in vitro in buffalo cows. Thus, the practical aim of this work was to study the possible causes of infertility and low fertilization rate in Egyptian buffalos as well as to know which stage of estrous cycle that a good quality oocytes could be obtained and matured in vitro.

MATERIALS AND METHODS

1. Ovaries collection and predicting stages of estrous cycle:

Forty six palirs of buffalo ovaries were collected from slaughter houses in Giza Province. Immediately after slaughtering, ovaries were transferred in a physiological saline at 30 to 35 ∞C, to the laboratory within 2 hours. The stages of the estrous cycle were determined according to the gross-appearance, weight and diameter of corpora lutea (number in stage I, 15; II, 17; III, 5; IV, 9) (El-Sawaf and Schmidt, 1962; Ireland et al., 1980; Eissa, 1996). Pair of ovaries (right and left) in each stage was prepared and examined histologically for follicular growth and atresia (Bancroft et al., 1994). Follicular fluid from medium size follicles (2-7 mm in diameter) from each ovary was collected for recovery of oocytes and for hormonal and biochemical analysis.

2. Oocyte recovery and in-vitro maturation:

The oocytes recovered from different stages were differentiated according to their quality into good (Cumulus Oocyte Complex "COC"; partially compact) and poor quality (expanded cumulus; denuded and degenerated). Good quality oocytes

were cultured in maturation media that consists of Ham's F10 medium (Sigma, No. 1387), supplemented with 10% heat inactivated fetal calf serum, 10 i.u./ml LH (Pregnyl-Nil Co., A.R.E.), 5 i.u./ml FSH (Folligon, Intervet); 1 µg/ml estradiol-17 B in ethanol (Sigma, E 8878), 10 µl/ml sodium pyruvate and 50 µg/ml gentamycin (Sigma, G 1264). Maturation was performed by culturing approximately 5-10 good quality oocytes in 50 µl droplet of maturation media under liquid paraffin oil and incubated in a humified atmosphere of 5% CO2 in air at 39 ∞C for 22-24 hours (Totey et al., 1993).

Maturation was evaluated by degree of the cumulus expansion according to Lorenzo et al. (1994), into complete expansion, unexpanded or degenerated oocytes. Moreover, evaluation of nuclear maturation was performed according to the method described by Richard and Sirard (1996) and Abbas (1998). They differentiated the nuclear maturation into: germinal vesicle stage (GV), first metaphase (MI), first anaphase (AI) and second metaphase (MII). The authors considered that oocytes become mature when achieved to the MII stage.

3. Estimation of follicular fluid steroid hormones and some biochemical constituents:

Samples of the follicular fluid were centrifuged at 3000 rpm for 15 minutes and the supernatants were kept frozen at - 20 °C untile analysis.

Concentration of steroid hormones (progesterone, estradiol-17B and testosterone) were estimated in samples diluted with PBS (100 folds) using RIA kits (DPC, Diagnostic Corporation, Los Angeles) at Atomic Energy Authority Lab. Some biochemical constituents of follicular fluids were determined: Total protein, albumin and globulin (Grant et al., 1978); total lipids (Chabrol and Charonnat, 1937); cholesterol and triglycerides (Abell, 1958); glucose (Caraway and Watts, 1987); sodium and potassium (Knudsen et al., 1979); Calcium and Inorganic phosphorus (Fraser et al., 1987); Transaminase enzymes "AST &ALT" (Henry, 1965); creatinine (Varely, ,1976) and urea (Fawcett and Sctt, 1960).

4. Data analysis:

The data were statistically analysed using Chi-square test (X2) to determine the difference between stages of estrous cycle (Snedecor and Cohran, 1982). Moreover, analysis of variance was carried out with the aid of the general linear model procedure of the statistically analysis system (SAS, 1987).

RESULTS AND DISCUSSION

The predicted stages of estrous cycle were confirmed by a significant variation ($P < 0.01$) in weight and diameter of CL (Table 1), obtained

by El-Sawaf and Schmidt (1962) and Eissa (1996) in Egyptian buffaloes. They recorded that the big variation in the weight and diameter of CL may be due to the different stages of estrous cycle in buffaloes. Also, accuracy of estimating stages of the estrous cycle was determined by the presence or absence of graafian follicle of > 10 mm in diameter at the stage III or IV. It is in accordance with the results of Ireland et al. (1980) who also found that weight of corpora lutea increased from day 1 to 17 of bovine estrous cycle, then declined thereafter. They mentioned that the correlation between estimated and actual days of the estrous cycle was 0.81.

The highest good quality of recovered oocytes (with complete or partially compact cumulus cells) was registered at stage III of estrous cycle (100%), in comparable with other stages (50, 42.9 & 42.9% at stages I, II & IV; respectively) as shown in table (2). It may be attributed to the higher activity of CL which produces higher levels of progesterone hormone (Table, 3). Moreno et al. (1992) demonstrated that pregnant cows produced a higher number of good quality oocytes, probabely due to higher progesterone level in the follicles. However, Das et al. (1996) suggested that the presence of CL yields a lower number of oocytes and a lower proportion of good quality oocytes in buffaloes. While, Boediono et al. (1995) recorded no difference in the mean number and quality of the oocytes per ovary between CL bearing and non CL bearing cow ovaries.

On the other hand, the poor quality oocytes recovered from stages I, II & IV of the cycle, may be interpreted by the occurrence of atresia in the ovaries at these stages as shown in figures 3, 4 & 6. Das et al. (1996) and Kumar et al. (1997) recorded that the number of follicles and yield of oocytes per ovary were less from ovaries bearing a CL in buffaloes. The atresia may be due to hypoxia which results in disruption of ionic gradients among granulosa cells (Guraya, 1979; Hirshfield, 1991). Bruck et al. (1996) mentioned that oocytes from atretic follicles are more likely to exhibit a misshape and/or dense ooplasm and an expanded, pyknotic cumulus.

Oocyte maturation is traditionally defined as those events associated with cumulus expansion and initiation of germinal vesicle breakdown and completion of first meiotic division. The present results demonstrate that the stages of the estrous cycle influenced the maturation rate of oocytes. The highest number of oocytes with complete cumulus expansion (100%) and which resumed meiosis and reached to MII (75%) was occured at stage III (day 11-17 of the estrous cycle). It may be also attributed to the activity of CL and the highest concentration of follicular progesterone at

this stage (Table, 3). In parallel with the present results, Fishel et al. (1983) found that progesterone concentration was positively correlated with oocytes which matured and fertilized in vitro. Moreover, Boediono et al. (1995) determined that higher blastocytes production in vitro could be achieved from oocytes obtained from cows in luteal phase compared to follicular phase and from ovaries bearing an active CL. Machatkova et al. (1996) detected that a significantly higher proportion of bovine oocytes developed into blastocyts following isolation on day 14 to 16 than other days of estrous cycle. On the contrary, Leibfried and First (1979) and Suss et al. (1988) reported that the ability of bovine oocytes to undergo a nuclear maturation in vitro after removal from vesicular follicle is not dependent on either size of follicle or stages of estrous cycle. They added that the presence of some type of follicular investment, intact cytoplasm and dictyate stage chromatin may be the only determination of bovine oocyte's ability to mature in vitro when removed from cyclic cows.

The lower rates of oocyte maturation occurred in stages I, II & IV of estrous cycles (Table, 2) may be due to a high level of oocyte maturation inhibition (OMI) or inhibin which are negatively correlated with follicular progesterone level (Schwartz and Channing, 1977). They added that inhibin was decreased at days 10, 15 and 18 of the cycle. Channing et al. (1982) mentioned that OMI and inhibin have a major role in nuclear maturation of bovine oocytes and they are independent to the stage of estrous cycle. Moreover, Ireland and Roche (1982) suggested that a higher follicular P: E ratio, which is representive of luteinization or atretic follicle would be expected to contain low levels of OMI. Furthermore, Tsafriri et al. (1982) and Grimes and Ireland (1986) examined that meiosis is often resumed in atretic follicles and this is due to reduction in OMI level or perturbation of cumulus cell-oocyte communication which might be essential for inhibition of meiosis.

Table (3) demonstrated that intra-follicular progesterone and estradiol levels started to increase at stage II (day 5-10), peaked at stage III (day 11 - 17) and then sharply decreased as estrous progressed. While, the ratio between progesterone and estradiol values (P/E2) was at its optimum during stage III of the cycle as occured in the ovulatory follicle before ovulation (Ireland and Roche, 1982). Increases in P: E ratio may be related to the ability of endometrium to release luteolytic pulses of PGF2 ((Moore et al., 1980), which decreased progesterone level and increased aromatase activity and E2 production. Grimes and Ireland (1986) suggested that high progesterone and low estradiol levels in cows associated with the ability of oocytes to mature in vitro. Thus, it is assumed that the ratio of P> E in follicular fluid in

the present results, would be associated with the recorded nuclear maturation of oocytes at least for ovulatory follicles. However, Parmar and Mehta (1994) found that a lower estradiol and higher progesterone levels in follicular fluid during summer may not be conductive its optimal oocyte maturation and ovulation in buffaloes.

On the other hand, increased capacity of follicles to secrete progesterone at stage III may lead them to on going atresia (Fig., 5). It is shown atretic graffian follicles after the regression of the granulosa cell layers and also atretic premordial follicles were occurred in a diffuse manner allover the ovarian tissue. McNatty et al. (1984) and Wise (1987) suggested that the atresia could be seen at any stage of follicular development and this is due to the imbalance of FSH and LH. Moreover, Danell, 1987 and Driancourt et al. (1991) reported that low turnover of the graffian follicles and high frequency of atresia may affect hormone production, which leads to low reproductive efficiency in buffaloes as compared to that of cattle (Bharadwaj and Roy, 1998). In addition, it may be attributed to a decrease in activity of an enzyme that degardes progesterone, such as 20 (or 20 (hydroxysteroid dehydrogenase (Hsueh et al., 1984); or due to lower aromatase activity (Tsafriri and Brawn, 1984). Ireland et al. (1979) recorded that increasing in atresia was mostly occured during mid-cycle in cow. Moreover, Guthrie and Cooper (1996) found that incidence of atresia increased between day 5-7 of the cycle (46-50%) in medium sized follicles and reached peak at mid-cycle. They added that rising of progesterone level on day 17 than expected in normal estrous cycle could contribute to decrease fertility in cattle that result a prolonged dominant follicles or a prolonged luteal phase (Vande Weil et al., 1983).

Intra-follicular testosterone concentration increased markedly during stage IV (day 18-21) and peaked at stage I (day 1-4). It is in accordance with results obtained in buffaloes (Eissa, 1996) and in cows (Wise, 1987; Badinga et al., 1992), in which they reported that testosterone was the predominant androgen in the follicular fluid in early stage of the cycle (day 2-5) and it decreased with follicular development. On the contrary, Kanchev et al. (1976) mentioned that testosterone was present in much higher concentration than E2 during the estrous cycle in cows and peak of testosterone was detected at day 5-8. It is suggested that the higher testosterone concentration in the present results may be a characteristic feature of follicular atresia, particularly at stages I & IV of the cycle (Fig., 3 & 6). It may related to degeneration of granulosa cells and hypertrophy of thecal layers (Hillir and Ross, 1979), as the follicles may have blocked ability to respond to gonadotropin before preovulatory gonadotropin surge. Furthermore, Driancourt and Thuel (1998) recorded that

testosterone has an inhibitory effect on nuclear and cytoplasmic maturation and this is what happened in in-vitro maturation of oocytes recovered from stages I, II & IV in our study.

Since the follicular fluid is in intimate contact with the oocyte and granulosa cells cyclic changes in the concentration of its different components may reflect their roles in the requirements of follicular structure. The significant ($P < 0.01$) high level of follicular biochemical constituents (Table, 3) at stage III of the cycle, which may correlated with the quality and maturation of recovered oocytes, were total protein, albumin, globulin, triglycerides, sodium, potassium, calcium and inorganic phosphorus. Total protein in follicular fluid can probably maintain a colloid osmotic pressure equal to serum (Anderson et al., 1976). In parallel with our results, Shalgi et al. (1972) & Eissa (1996) in buffaloes and Wise (1987) in cows revealed that concentration of the protein increased as follicle developed. The significant increase of sodium and potassium at stage III were in agreement with results obtained in cows by Burgoyne et al. (1979) and Knudsen et al. (1979). It may be related to follicular viability and probably linked to active follicular synthesis of estrogen. Therefore, they used for the maintenance of oocytes in culture media (Shalgi et al., 1972; Powers and Biggers, 1976). McGaughey (1977) found that a hyperosmolarity of porcine follicular fluid can cause poor oocyte maturation in in-vitro culture. Intra-follicular calcium and inorganic phosphorus increased as the cycle progressed and then declined after 18 days of the cycle, coincide with the results reported in buffaloes by Eissa (1996). The calcium may play a crucial role in steroidogenic capability of growing follicles (Wise, 1987). Since calcium was a key nutrients in proliferative cells (granulosa and theca).

On the other hand, intra-follicular total lipids, cholesterol, creatinine, urea and transaminase enzymes (AST & ALT) were significantly decreased during stage III of the cycle. Lipids play a role in steroidogenesis and steroid content of the follicular fluid (Short, 1964) and cholesterol is a major precursor of steroid hormones (El-Sawaf and Schmedit, 1962). Therefore, there is a negative correlation between their levels and follicular progesterone concentration (Zimbelman et al., 1961). However, Mares et al. (1962) demonstrated that follicular cholesterol increased with increasing follicular progesterone to day 15 but continued to increase at day 17 and 19 after the progesterone had dropped and differences among stages were not significant. Transaminase enzymes may play a role in cell metabolism through their involvement in the vital cellular process. So, they significantly decreased with follicular development.

Glucose as a source of energy was significantly increased during stage II (day 5-10). It is in full agreement with results of Eissa (1996), who found in Egyptian buffaloes that glucose leve was maximal before estrous.

Table (1): Predicted stages of the estrous cycle in buffalo cows (Mean ± S.E.).

Stages	No. of Pairs of ovaries	Gross appearance of CL	Weight CL (g)	Diameter of CL (cm)	Follicle > 10 mm in diameter
I (day 1-4 Metestrous	15	- Red, recently ovulated, point of rupture not covered by epithelium. -Internally, red in color filled with blood - Vasculature on the surface of CL is not visible	0.9 ± 0.0^{c}	1.1 ± 0.1^{bc}	Absent
II (days 5-10) Diestrous	17	- Point of rupture covered by epithelium. apex of CL brownish in color - Vasculature on the surface of CL generally limited to the surface of CL	1.6 ± 0.1^{b}	1.2 ± 0.1^{b}	Present
III (day 11-17) Proestrous	5	- Grayish white in color - Same as II but it will cover apex of CL late in this stage.	2.7 ± 0.2^{a}	1.9 ± 0.1^{a}	Present or absent
IV (day 18-21 Oestrous	9	- Scar formed connective tissue spread witin the matrix of the ovarian tissue, pale brown in color - Vasculature on the surface of CL not visible.	1.0 ± 0.1^{c}	1.0 ± 0.1^{c}	Present

Means in the same column with the different superscripts were significantly differ at $P < 0.01$.

Table (2): Quality and in-vitro maturation of recovered oocytes from ovaries at different stages of estrous cycle in buffalo cows.

	Stages of estrous cycle				Follicle > 10 mm in diameter
	I	II	III	IV	
1. Quality of oocytes					
Number of oocytes	60	70	20	35	
1. Good quality					
Compact	30	20	15	10	
Partial compact	-	10	5	5	
Total (%)	30 (50)	30 (42.9)	20 (100)	15 (42.9)	5.656
II. Poor quality					
Expanded	5	-	-	10	
Denuded	20	20	-	5	
Degenerated	5	20	-	5	
Total (%)	30 (50)	40 (57.1)	-	20 (57.1)	10.736**
2. In-vitro maturation					
Number of good oocytes	30	30	20	15	
I. Degree of cumulus expansion					
Complete	15 (50)	20 (66.7)	20 (100)	10 (66.7)	2.462
In complete	15 (50)	10 (33.3)	-	5 (33.3)	8.498*
Degenerated	-	-	-	-	-
II. Nuclear maturation					
Germinal vesicle	-	-	-	-	
Metaphase I	-	-	-	-	
Anaphase I	15 (50)	15 (50)	5 (25)	5 (33.3)	1.910
Metaphase II	15 (50)	15 (50)	15 (75)	10 (66.7)	1.128
Degenerated	-	-	-	-	

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

Table (3): Hormonal and biochemical constituents of follicular fluid of buffalo-cows during different stages of estrous cycle.

Constituent	Stages of estrous cycle				F. value
	I	II	III	IV	
No. of samples	5	5	5	5	
Progesterone (ng/ml)	60.0 ± 1.7^{c}	200.0 ± 1.7^{b}	1680 ± 8.0^{a}	190.0 ± 2.2^{b}	2756.994**
Estradiol (pg/ml)	315 ± 3.5^{dc}	360 ± 3.5^{b}	420 ± 3.5^{a}	320 ± 3.5^{c}	188.5**
P:F. ratio	0.19 ± 0.02^{b}	0.53 ± 0.02^{b}	4.0 ± 0.40^{a}	0.63 ± 0.02^{b}	99.54**
Testosterone (ng/ml)	20 ± 0.2^{a}	11.7 ± 0.1^{e}	3.2 ± 0.1^{d}	15.6 ± 0.2^{b}	2317.2**
Total protein (g/100 ml)	59.9 ± 0.3^{d}	68.8 ± 0.3^{b}	72.2 ± 0.6^{a}	68.6 ± 0.2^{bc}	156.61**
Albumin (g/100 ml)	7.9 ± 0.5^{d}	16.4 ± 0.2^{cb}	18.3 ± 0.2^{a}	17.1 ± 0.1^{b}	490.20**
Globulin (g/100 ml)	52.0 ± 0.0^{bc}	52.4 ± 0.1^{b}	53.9 ± 0.1^{a}	51.5 ± 0.1^{bcd}	13.72**
Total lipids (g/100 ml)	283.4 ± 1.5^{a}	285.3 ± 3.6^{a}	227.5 ± 1.7^{c}	258.3 ± 1.3^{b}	149.51**
Cholesterol (g/100 ml)	150.8 ± 6.7^{a}	103.1 ± 4.6^{b}	66.8 ± 3.0^{d}	93.6 ± 4.2^{bc}	52.76**
Triglyceride (g/100 ml)	159.6 ± 0.5^{c}	173.0 ± 0.7^{a}	175.3 ± 0.4^{a}	164.0 ± 0.7^{b}	159.5**
Creatinine (mg/dl)	2.8 ± 0.1^{a}	2.1 ± 0.1^{b}	1.3 ± 0.1^{bc}	3 ± 0.2^{a}	6.6**
Urea (mg/dl)	35.1 ± 0.3^{b}	34.9 ± 0.3^{bc}	30.5 ± 0.3^{d}	46.9 ± 0.1^{a}	204.33**
Sodium (mmol/L)	130 ± 3.5^{d}	148 ± 2.3^{c}	210 ± 3.5^{a}	195 ± 1.8^{b}	170.38**
Potassium (mmol./L)	24.3 ± 0.2^{c}	19.5 ± 0.4^{d}	46.1 ± 0.1^{a}	26.6 ± 0.2^{b}	3423.3**
Calcium (mg%)	7.5 ± 0.4^{dc}	8.6 ± 0.3^{b}	9.5 ± 0.1^{a}	8.2 ± 0.3^{bc}	6.96**
Phosphorus (mg%)	5 ± 0.2^{bc}	6.1 ± 0.2^{b}	7.9 ± 0.2^{a}	5.7 ± 0.2^{d}	44.98**
AST (U/L)	20.1 ± 0.1^{b}	21.6 ± 0.2^{a}	13.1 ± 0.1^{d}	17.8 ± 0.1^{c}	574.03**
ALT (U/L)	44.6 ± 0.3^{b}	55.2 ± 0.2^{a}	33.3 ± 0.2^{d}	40.7 ± 0.2^{c}	742.18**
Glucose (mg%)	2.5 ± 0.4^{dc}	6.0 ± 0.3^{a}	4.5 ± 0.3^{b}	3.0 ± 0.4^{c}	19.84**

**Means in the same raw with the different superscripts were significantly differ at $P < 0.01$.

** : $P < 0.01$.

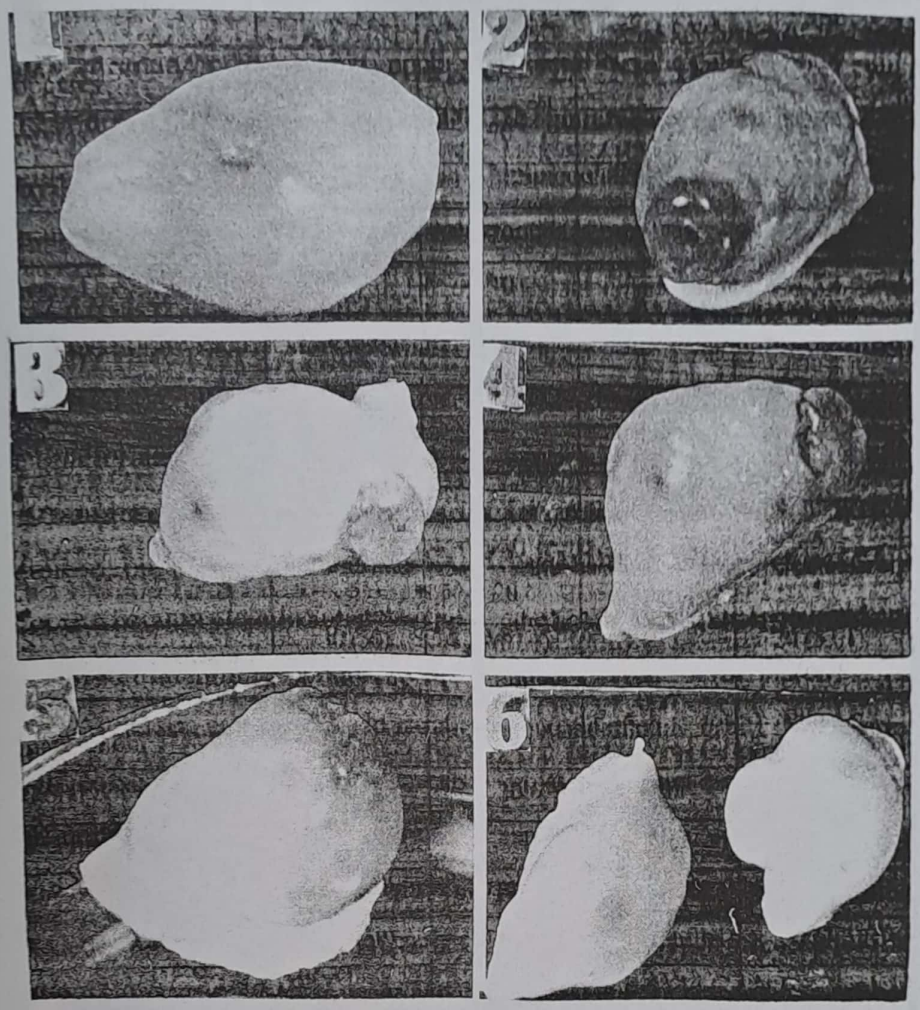

Fig. (1): See table 1, for description of ovaries, I through IV.

1 & 2 Stage I.

3 & 4 Stage II.

5 Stage III.

6 Stage IV.

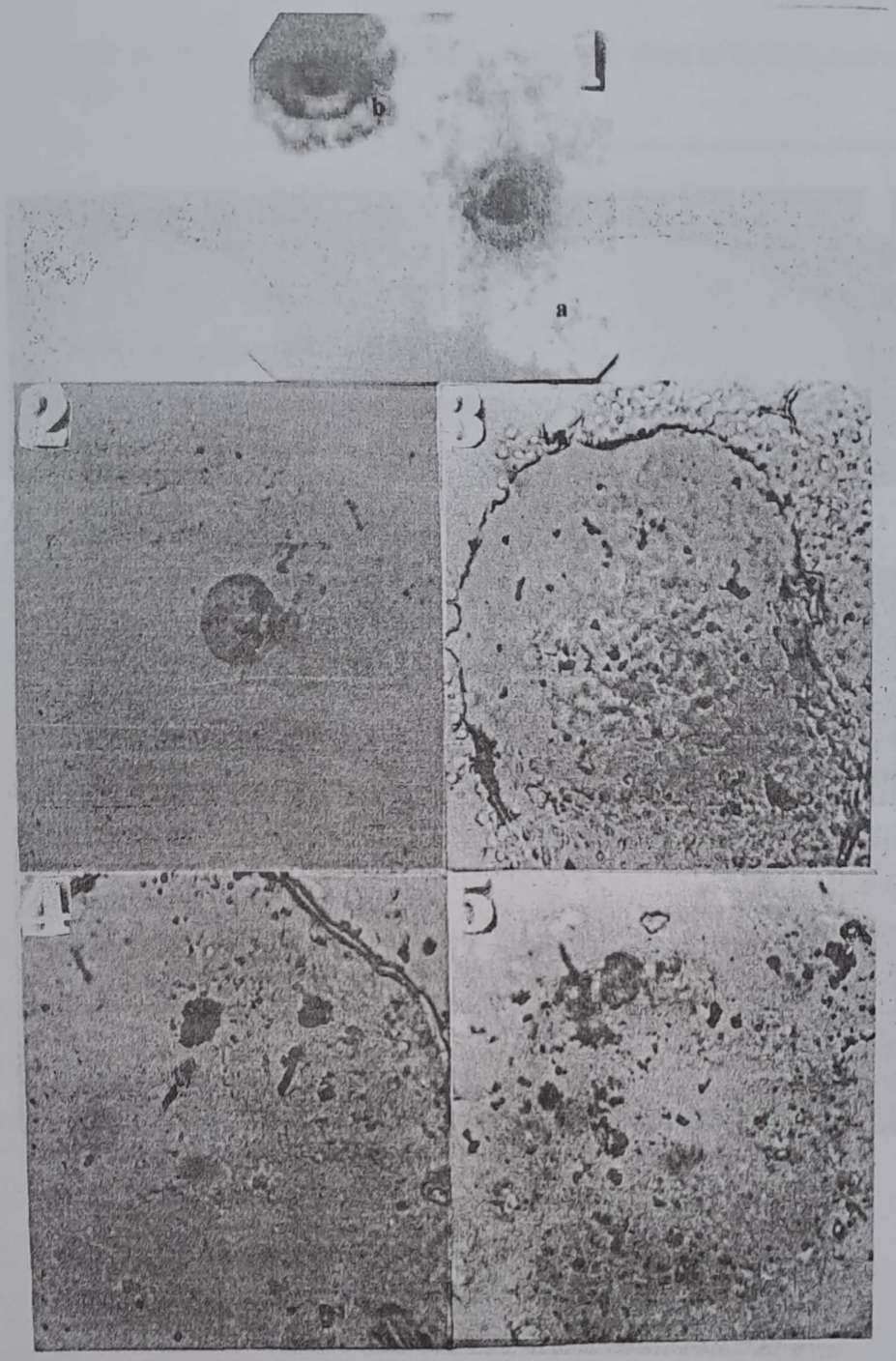

Fig. (2): Showed:

1. Mature buffalo oocyte showing either complete (a) or incomplete (b) expanded cumulus.
2. Oocyte with germinal vesicle stage, showing an intact nuclear membrane with the chromatin of meiotically inactive.
3. Oocyte in metaphase I stage, showing the nuclear membrane was broken and paired chromosomes or bivalents were observed.
4. Oocyte in anaphase I stage, showing separation of homologous pairs of chromosomes. The chromosomes were pulling a part from each other and moving to opposite poles of the spindle.
5. Oocyte in metaphase II stage showing (a) polar body chromosomes and (b) metaphase chromosomes.

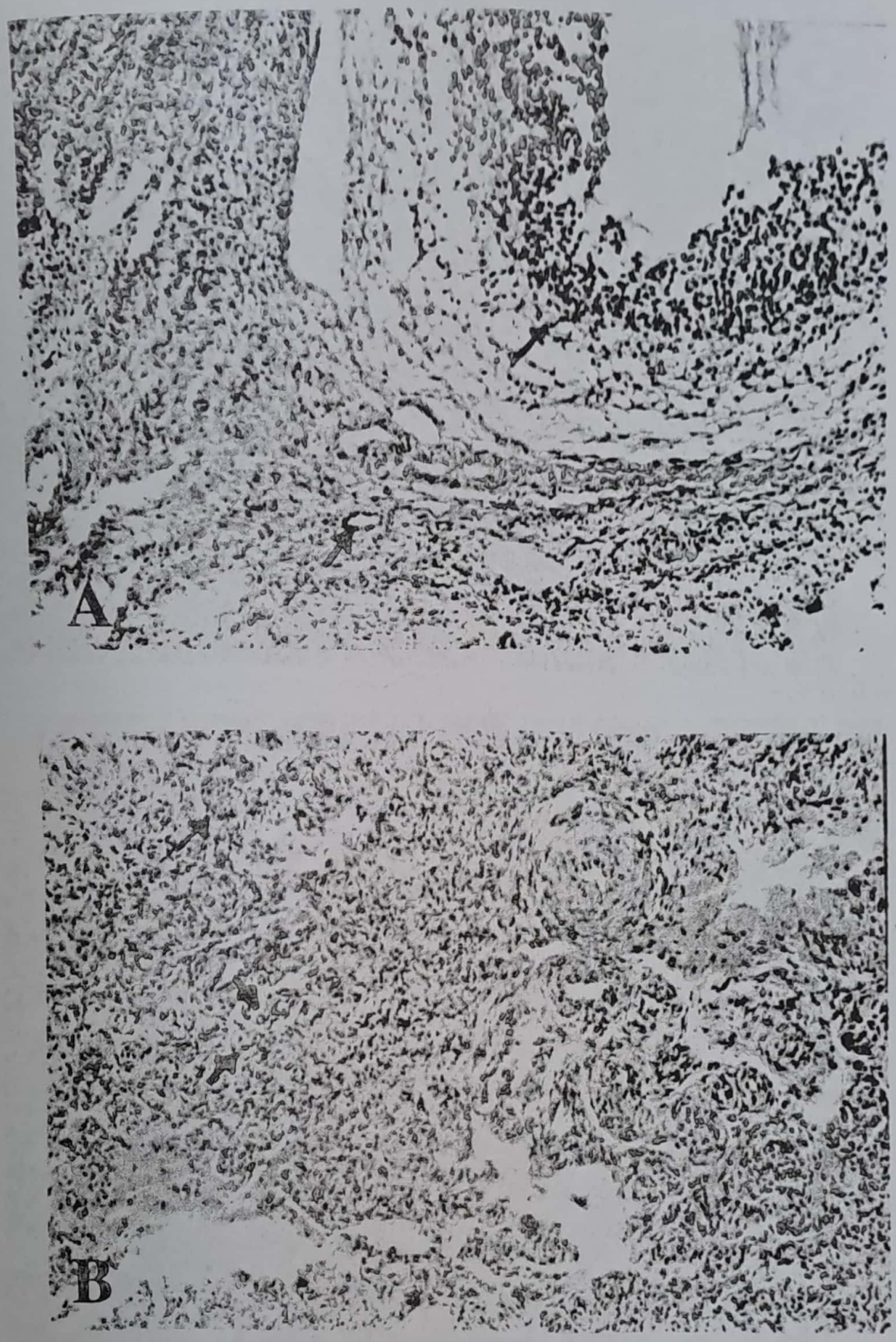

Fig. (3): Ovaries in met-estrous (Stage I) showing (a): Degenerative change of the granulosa cells and a part of graffian follicle in the highly cellular cortex with atresia of other follicles (right side) and (b): Atresia of the follicles with appearance of yellow golden pigments were replaced the ovarian tissue in most cortical and medullary portion (left side). (H & E. X: 40).

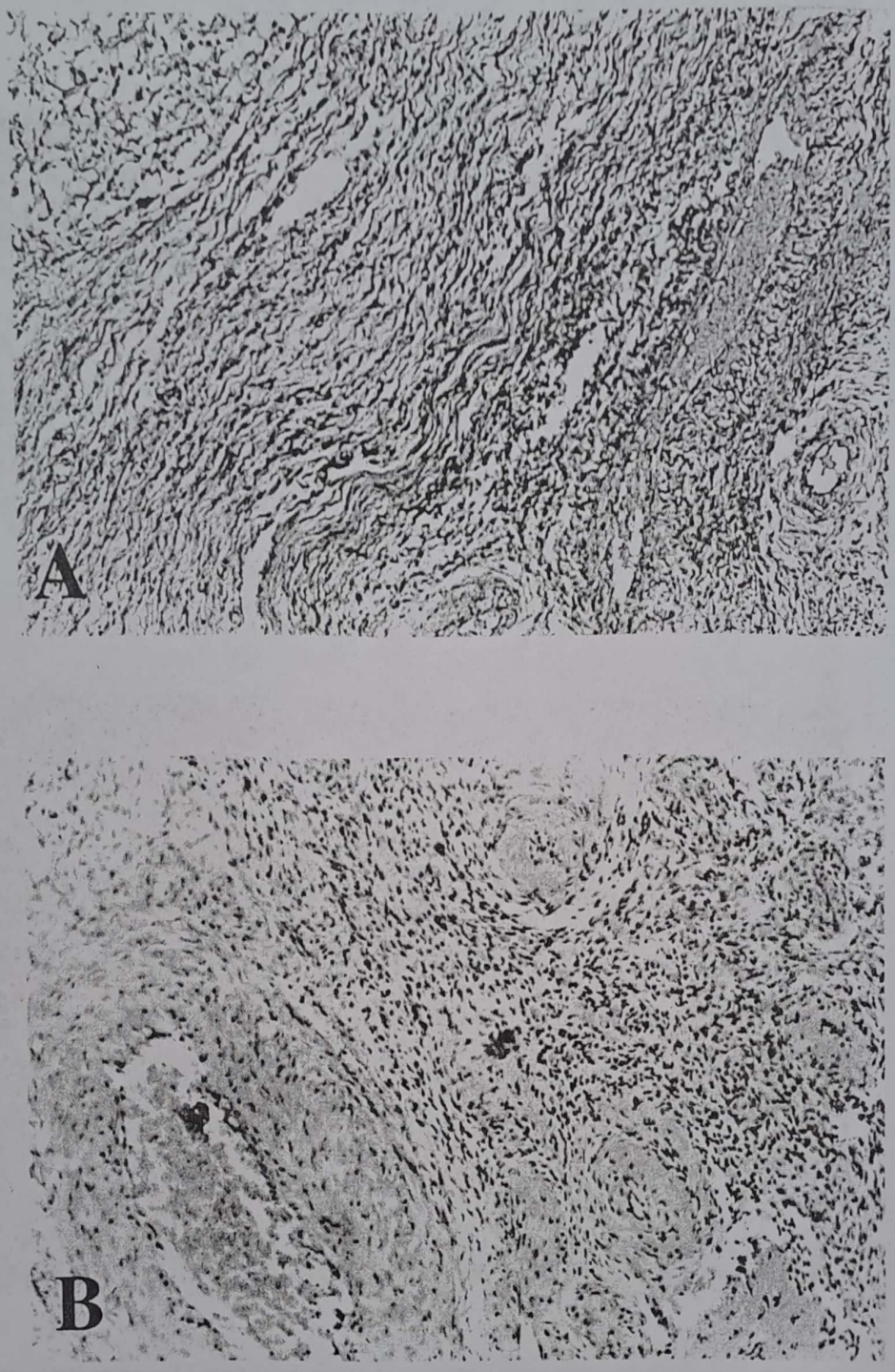

Fig. (4): Ovaries in diestrous (Stage II) showing, (a): Highly fibrillar part of the medullary portion with a cortical portion containing corpus albican with complete atretic follicle (right side). (b): Atresia of the primordial follicles with absence of the graffian one also showing a port of cortical cellular portion with dilated blood vessels and some focal yellow pigment (left side). (H & E. X: 40).

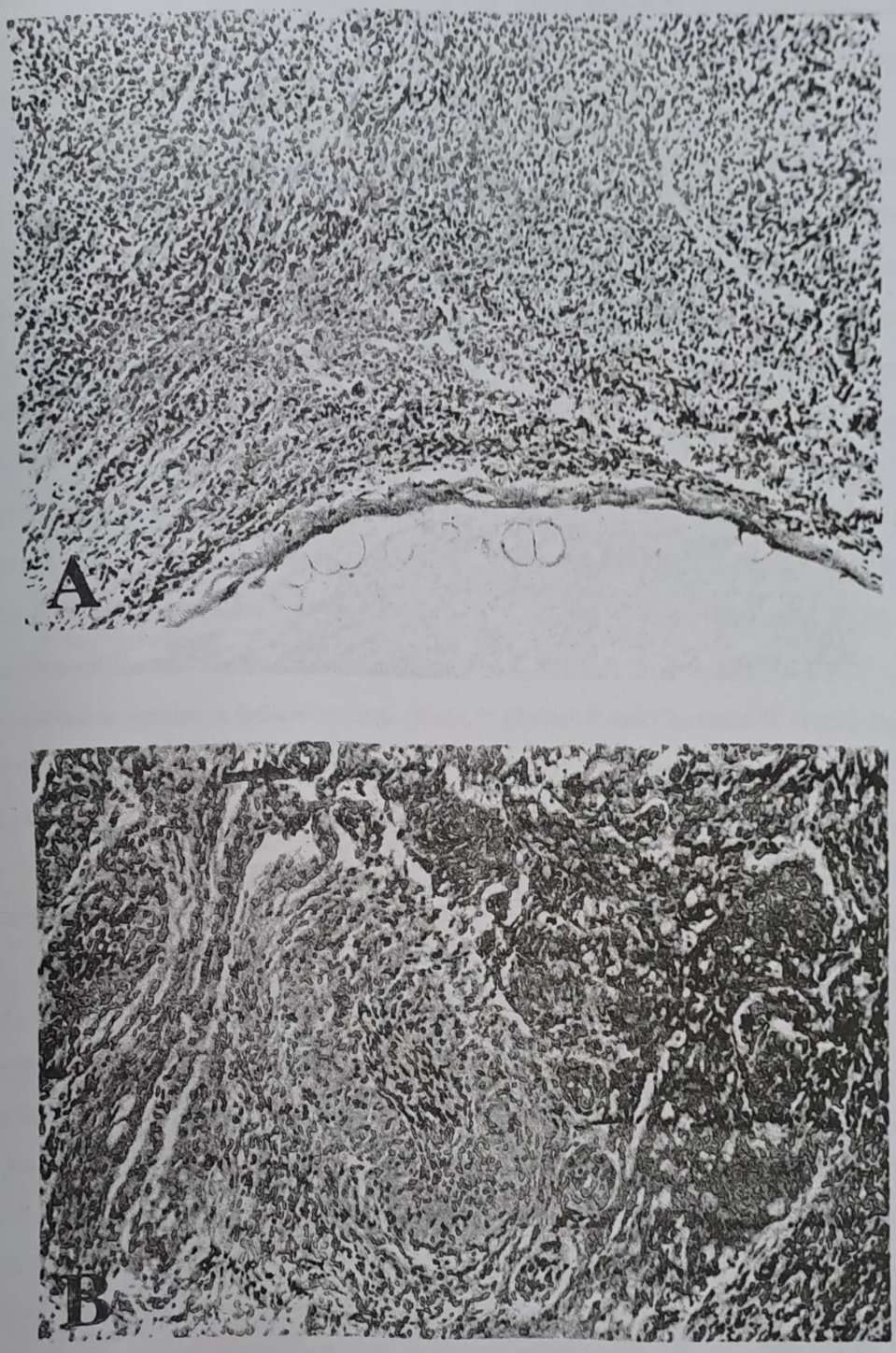

Fig. (5): Ovaries in proestrous stage III showing, (a): The wall of follicular cyst Liquor in the highly cellular cortex which replaced the atretic graffian follicle after the regression of the granulosa cells layer (right side). (b): :Sclerotic blood vessels with atretic primordial follicles in the fibrillar medullary portion (left side). (H. &E. X: 40).

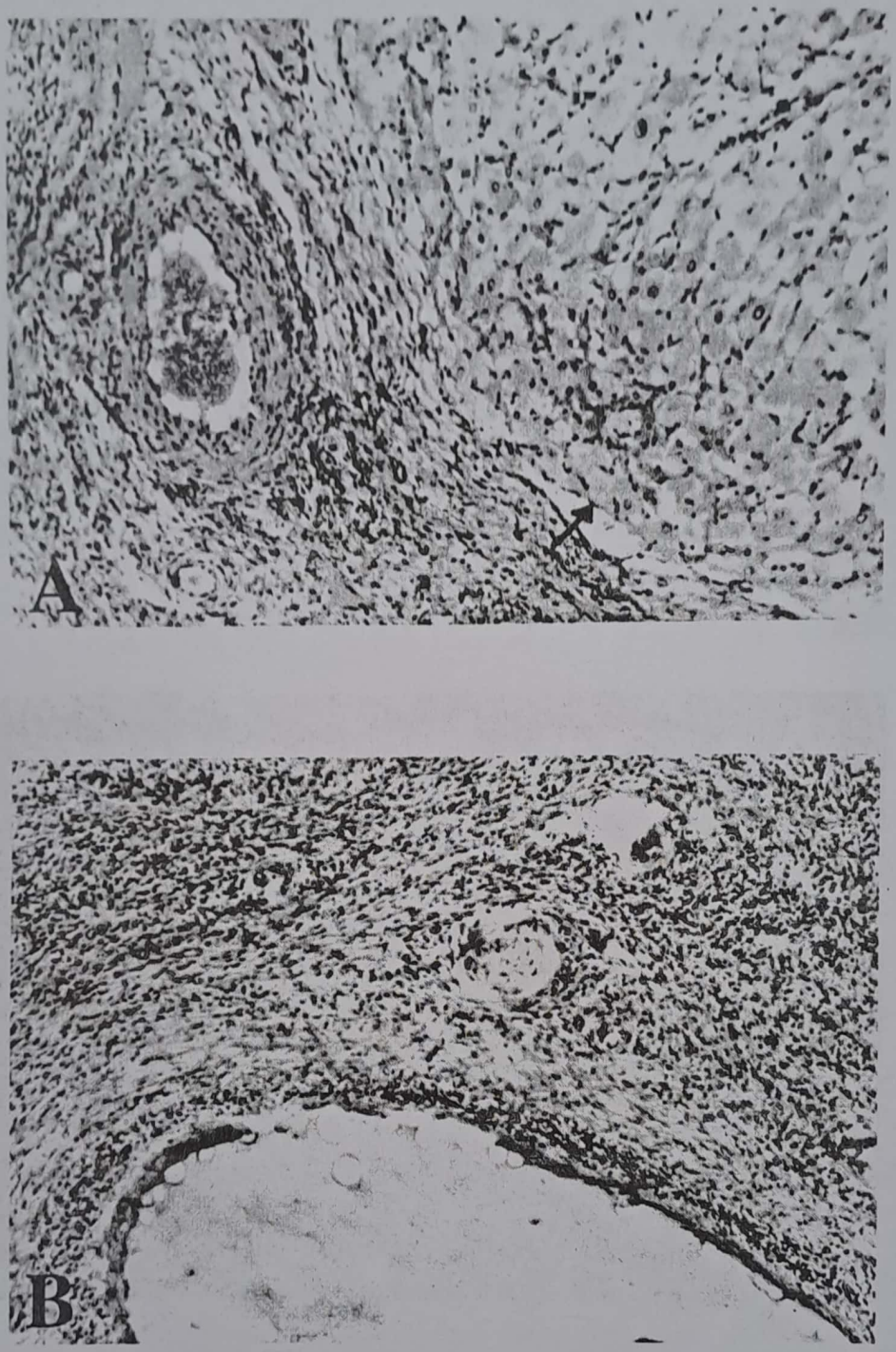

Fig. (6): Ovaries in estrous stage IV showing, (a): A corpus luteum which replaced most of the ovarian structure with complete absence of the mature graffian follicles as well as atresia of the primordial one (right side). (b): A follicular cyst formation with atresia of others (left side). (H. & E. x: 40).

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