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A MORPHO-HISTO-ENDOCRINOLOGICAL STUDY OF OVARIAN FOLLICULAR DEVELOPMENT IN BUFFALO-COWS

(With 3 Tables and 2 Figures)

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د ر ا سة مورفولوجيه ، هستولوجيه وهرمونيه لنمو الجريبات المبيضيه فى الجاموس

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الهــدف من إجراء هذا البحث هو مناقشة نظرية فرضية مفادها أن الجر يبات المبيضيه في الجــاموس المصري تنمو على شكل موجات متتابعة. أجرى الجزء الأول من الدراسة على عدد ٢٠٠ جهاز تناسلي. تم تقسيم دورة الشبق بناء علي الشكل الظاهري للجسم الأصفر إلى ٤ فترات: من اليوم ١-٤ ؛ من اليوم ٥-١٠ ؛ من اليوم ١١-١٧و من اليوم ١٨-٢١. وفى هــذا الجــزء مــن البحث تم تحديد مكان الجريب السائد هل هو في المبيض الأيمن أو في المــبيض الأيسر خلال فترات دورة الشبق المختلفة؟. في الجزء الثاني تم فحص الجر يبات السائدة هستولوجيا"خلال فترات دورة الشبق المختلفة في عدد ٣١ عينه. فـــي الجزء الثالث (٢٣ عينه) تم تحليل سائل الجر يبات السائدة لقياس هرموني الا ستروجين و البروجستيرون خـــلال فترات دورة الشبق المختلفة. أوضحت النتائج ما يلي: (١) غير الجريب السائد مكانه مـــن فترة إلى أخرى خلال فترة الشبق. (٢) في الفترة من اليوم ٥-١٠من دورة الشبق كان الجريب السائد في اغلب الحالات نشيطا" هرمونيا" و سليما" هستولوجيا". (٣) في الفترة من اليـوم ١١-١٧ كــان الجــريب الســائد فقط في نصف الحالات نشيطا" هرمونيا" و سليما" هســتولوجيا". (٤) فـــي نهاية دورة الشبق (من اليوم ١٨-٢١) كان الجريب السائد نشيطا" هرمونيا" و سليما" هستولوجيا" في كل الحالات. هذه الظواهر مجتمعة من تغير مكان و نشاط و سلامة الجريب السائد من فتره إلى أخرى خلال دورة الشبق يعضد النظرية الفرضية بأن الجر يبات المبيضيه في الجاموس المصري تنمو على شكل موجات متتابعة.

SUMMARY

The aim of the present study is to test the hypothesis of the wave pattern of follicular growth in the Egyptian buffalo-cows. In the first experiment the genital tracts of 200 buffalo-cows were collected from a local abattoir. According to morphological appearance of the corpus luteum, estrous cycle was divided into 4 stages: I (d1- d4), II (d5 - d10), III (d11 - d17), and IV (d18 - d21), follicular populations were counted and location of dominant follicle (DF) whether on the right or left ovary was recorded. In the 2nd experiment, ovaries from 31 other organs and bearing DF during stages II, III and IV were processed histopathologically to evaluate the atretic status of the DF. In an extra 9 cases during mid-cycle, the larger and sub-larger follicles were evaluated histopathologically. In the third experiment, follicular fluids (FF) of 23 DF during stages II, III and IV were aspirated and analyzed for estradiol 17β (E_2) and progesterone (P_4) using RIA. In a further 10 cases the two largest follicles were also analyzed for E_2 and P_4. The results showed that, DF changed its position from one stage of the estrous cycle to the other. DF between d5 and d10 was in the most cases functionally active (E_2-dominant,) and non-atretic. However, half of the DF in next stage (d11-d17) became functionally inactive (P_4-dominant) and atretic. Towards the days of proestrus and estrus, DF became in all cases functionally active and non-atretic. In the majority of specimens that carried two large follicles, one follicle was atretic and the other was non-atretic. Hormonal analysis of the two large follicles revealed that, in most cases both follicles were different in the hormonal status. It could be concluded that, stage-related differences in location, atretic status and hormonal contents of dominant and subordinate follicles support the hypothesis that follicular development in the Egyptian buffalo-cow occurs in a wave-like pattern. The majority of cows showed two wave-like patterns, however one wave pattern was detected in some cows.

Key words: dominant follicle, atresia, estradiol, progesterone, buffalo.

INTRODUCTION

Buffaloes constitute a large scale of animal population in Asian and Mediterranean areas (about 150 Million). Also, there is an increasing demand to rear buffalo in some of the European lands (Kruel, 1991 and Ali *et al.*, 2000). However, fertility in buffalo is yet much lower than that in cattle (Dobson and Kamonpatana, 1986 and Singh *et al.*, 2000). Indeed, beside the genetic ground for this difference (Danell, 1987), there are also managerial cause (Staigmiller and England, 1982). Weak estrous signs, silent heat, seasonal anestrum and the long postpartum anestrum are considered the major reason of this subfertility in buffaloes (Gordon, 1997 and Singh *et al.*, 2000).

To improve fertility in buffalo, first of all, an adequate knowledge about the reproductive cycle is necessary. To establish the most modern programs of estrous synchronization and superovulation in buffalo industry, a full information about follicular development and dynamics is essential. Unlike cattle, little attention has been focused on the follicular development in buffalo.

Follicular atresia has been described as a normal phenomenon in the ovary (Rajakoski, 1960). It has been described in several mammalian species and has been covered in the literature for cattle (Rajakoski, 1960) and buffalo as well (Danell, 1987). In cattle, follicles develop in a wave like pattern, with two or three follicular waves per cycle (Savio *et al.*, 1988 and Sirios and Fortune, 1988). Only the dominant follicle of the last wave has the chance to ovulate, while the other follicles undergo atresia. Histopathological examination has been used to differentiate between atretic and non-atretic follicles (Rajakoski, 1960 and Danell, 1987), so it could be utilized to study the turnover of follicles during estrous cycle.

The capacity of follicles to synthesize estrogen is a function whether that follicle is atretic or not (Ireland, 1979). Degeneration of the follicles is accompanied by a decrease in estradiol 17β (E_2) concentration and by increase on progesterone (P_4) content (Kruip and Dieleman, 1985). Except in preovulatory follicle a high P_4 concentration is a parameter of atresia. E_2/P_4 ratio has been utilized to distinguish between functional active and inactive follicles (Ali *et al.*, 1999). Thus, estimation of E_2/P_4 in the follicular fluid (FF) of large follicles could be used to follow up their developmental status during different stages of cycles.

In cattle follicular dynamics have been intensively studied (Rajakoski, 1960; Matton *et al.*, 1981; Savio *et al.*, 1988; Sirios and Fortune, 1988; Adams, *et al.*, 1992 and Ginther *et al.*, 1996). Similarly, there are some reports about follicular development in the Indian Murrah (Manik *et al.*, 1994; Taneja *et al.*, 1996 and Baruselli *et al.*, 1997) and Surti (Danell, 1987) buffaloes. However, follicular dynamic in the Egyptian buffalo has not yet been investigated. So, the aim of the present work was to test the hypothesis of wave pattern of follicular growth in the Egyptian buffalo-cows using a morpho-histo-endocrinological model.

MATERIAL AND METHODS

First experiment (A):

Genital tracts of 200 normal cyclic Egyptian buffalo-cows (*Bubalus bubalis*) aging between 6 and 10 years old were collected at a local slaughter house. According to morphological appearance of the corpus luteum, the estrous cycle was divided into 4 stages (Jainudeen, *et al.*, 1983). Stage I (metestrus, d1 to d4) includes interval between ovulation and the time when epithelium grows over the rupture point. During Stage II (early diestrus, d5 to d10) the corpus luteum is fully formed with vasculature visible around its periphery, the apex is red or brown and the remaining is grayish. Stage III (late diestrus, d11 to d17) begins when the red or brown color disappears leaving the entire corpus luteum bright red or gray. During stage IV (proestrus and estrus, d18 to d21) the regressed corpus luteum appears small, hard, bright and with no vasculature visible on its surface.

Follicular population was counted, measured with a caliper to the nearest millimeter and classified into 4 classes (>12 mm, 9-12 mm, 5-8 mm and <5 mm in diameter).

Dominant follicle (DF) was defined morphologically as that more than 8 mm in diameter and exceeded the diameter of other follicles. Location of DF during different stages of the estrous cycle was recorded.

Second experiment (B):

The material for this experiment was procured from the genitalia of 31 buffalo cows other than those in the previous experiment. Shortly after slaughter, ovaries bearing DF (n=31) during stage II (n=10), stage III (n=16) and stage IV (n=5) of estrous cycle were fixed in 10% neutral formalin. The fixed ovaries were dehydrated in alcohol, processed and embedded in paraffin blocks. Sections of 5-7 μ were prepared and stained with heamatoxylin and eosin stain (Bancroft and Stevans, 1982). Dominant follicles were classified as normal or atretic after the criterion described by Danell (1987): Normal DF showed intact and healthy cumulus and/or granulosa cell layers as well as intact theca interna and externa layers (Fig. 1). In the atretic follicle, the cumulus and/or granulosa cell layers disappeared and sometimes, a marked connective ingrowth lined the antrum (Fig. 2).

In 9 cases where two large follicles more than 8 mm were found in the same specimen during late diestrus (d11 and 17), both follicles were processed histpathologically to show the level of atresia in the both follicles.

Third experiment (C):

In this experiment, follicular fluids (FF) was collected from 23 DF belonging to 23 genitalia, other than those induced in experiment A and B. These follicles were distributed in the different stages of estrous as follows: stage II (n=5), stage III (n=14) and stage IV (n=4). The fluids were aspirated using a 5 ml syringe connected with a 18G needle. After dilution of the FF 1:100 with Nacl 0.09%, it was stored at -20° till analysis. The FF were analyzed for E_2 and P_4 using a RIA technique as modified by Glatzel and Schallenberger (1990). Coefficient of variance of intra- and interassay were 12.3 and 12% for P_4; 8.5 and 14.8% for E2 respectively. Cross-reaction of antibodies against P4-estimation was less than 2.9% (testosterone) and against E_4-estimation less than 2.5% (estron).

In 10 cases where two follicles more than 8 mm were present in the same specimen between d11 and 17, both follicles were aspirated (n=20 follicles) and their FF were analyzed separately for E_2 and P_4 using the same previous technique.

Differences between number of follicles during different stages of estrous cycle were compared with the analysis of variance (ANOVA). Frequencies were compared with Chi-square test. Goodness -of fit Chi-square analysis were used to detect frequencies that different from equality. Data were considered to be significantly different at $p < 0.05$.

RESULTS

The average number of follicles of different sizes during the different stages of estrous cycle is presented in Table 1. Follicles smaller than 5 mm in diameter were higher during metestrus than during other stages of estrous cycle ($p<0.01$). Follicles between 5 and 8 mm in diameter were lower in number during proestrus and estrus than that during metestrus and late diestrus ($p<0.05$). Follicles 9-12 mm during early diestrus and late diestrus were more higher than that during metestrus, proestrus and estrus ($p<0.01$). Follicles larger than 12 mm was absent during metestrus and increased steady thereafter.

Table 1: Mean number of follicles during different stages of estrous cycle (Experiment A)

Stages of estrous cycle	Number of follicles (mean ± SD)			
	<5mm	5-8mm	9-12mm	>12mm
Stage I (metesrus, d1-d4) n=20	12.1^{a} ±8.3	2.35^{a} ±1.8	0.25^{a} ±0.1	0.00^{a} ±0.0
Stage II (early diestrus, d5-d10) n=45	6.29^{b} ±5.2	1.71^{ab} ±1.8	0.71^{b} ±0.6	0.24^{b} ±0.4
Stage III (late diestrus, d11-d17) n=110	5.93^{b} ±5.1	2.26^{a} ±2.8	0.64^{bc} ±0.8	0.55^{c} ±0.6
Stage IV (proestrus and estrus, d18-d21) n=25	7.4^{b} ±5.4b	1.28^{b} ±1.5	0.4^{ac} ±0.6	0.84^{d} ±0.6

[a,b,c,d] Columns with different superscript differ significantly.

The percentage of presence of large follicles (>8 mm) increased steady from 25% during metesrus to 100% during proestrus and estrus ($p<0.01$). The incidences of presence of two large follicles in the same specimen were 0, 11.1, 27.3 and 29% during metesrus, early diestrus, late diestrus and proestrus and estrus, respectively.

The DF was found during early diestrus on the right ovary in 34.9% and on the left one in 65.1% ($p<0.05$). During late diestrus it was located on the right and left ovaries in 53.4 and 46.7%, respectively. During proestrus and estrus it was found in 54.5 and 45.5% on the right and left ovaries, respectively.

Histopathological examination of 31 DF revealed that, 70% of the DF during early diestrus were non-atretic, while 30% were atretic. During late diestrus, 43.8% were non-atretic and 56.2% were atretic, while during proestrus and estrus 100% of the DF were non-atretic (Table 2).

Table 2: Incidence of atresia in the dominant follicle (DF) during different stages of estrous cycle (Experiment B).

Stages of estrous cycle	Incidence of atresia in DF	
	Non-atretic	Atretic
Stage II (early diestrus, d5-d10) N=10	$70\%^{ab}$	$30\%^{a}$
Stage III (late diestrus, d11-d17) N=16	$43.8\%^{a}$	$56.2\%^{a}$
Stage IV (proestrus and estrus, d18-d21) N=5	$100\%^{b}$	$0\%^{b}$

[a,b] Columns with different superscript differ significantly

Histological examination of the two large follicles present in the same specimen during late diestrus revealed that one follicle was atretic and the other was non-atretic in 7/9. The larger follicle was the atretic one in 3/7 cases.

E_2 concentration in the FF of the DF ranged between 0.5 and 60 ng/ml, while their P_4 content varied between 0.3 and 518.5 ng/ml.

Hormonal analysis of follicular fluid of the DF during different stages of estrous cycle showed that, DF during early diestrus were E_2-dominant in 80% and P_4 dominant in 20%. They were E_2-dominant in 50% and P_4 in 50% during late diestrus and they were 100% E_2-dominant during proestrus and estrus (Table 3).

Table 3: Incidence of dominant follicle with Estradiol 17β or Progesterone dominant during different stages of estrous cycle (Experiment C)

Stages of estrous cycle	Incidence of dominant follicle with	
	Estradiol dominant ($E_2/P_4>1$)	Progesterone dominant ($E_2/P_4<1$)
Stage II (early diestrus, d5-d10) n=5	80% [a]	20% [a]
Stage III (late diestrus, d11-d17) n=14	50% [b]	50% [b]
Stage IV (early diestrus, d5-d10) n=4	100% [a]	0% [a]

[a,b] Columns with different superscript differ significantly.

In case of presence of two large follicles more than 8 mm in the same specimen during late diestrus, both follicles were P_4-dominant in 2/10, while in 8/10, one follicle was E_2-dominant and the other was P_4-dominant. The larger follicle was E_2-dominant in half of the cases. In no instance both follicles were E_2-dominant.

DISCUSSION

The literature lacks proper investigation on the follicular development in buffaloes. Thus more effort have to be directed to understanding the process of follicular dynamics in buffalo. Although real time ultrasound is the ideal method which could give an accurate information about ovarian follicular dynamics, yet it could be used only on small number of animals (Manik *et al.*, 1994 and Taneja *et al.*, 1996). Also, it needs frequent or daily follow up for at least one cycle, needs expensive equipment and can not give information about the intrafollicular condition.

In the current study, follicles smaller than 5 mm in diameter were more numerous at the beginning of estrous cycle (between d1 and d4) than during other later stages of cycle, indicating that a follicular wave was recruited during this period. In cattle, several studies reported that the first follicular wave started around the day of ovulation and is characterized by recruitment of many small follicles at the same time (Ireland *et al.*, 1979; Savio *et al.*, 1988; Kahn, 1989 and Ali, 2000). Also, in Murrah buffalo the first follicular wave appeared approximately on the day of ovulation (Manik *et al.*, 1994 and Taneja *et al.*, 1996). The increase in number of small follicles at the beginning of estrous cycle might have resulted from the effect of the FSH-surge, which have been observed in the cow (Adams *et al.*, 1992 and Ginther *et al.*, 1996). This class of follicle (< 5mm) is reduced in number after day 4. Matton *et al.* (1981) observed in cattle that small follicles (1-3mm) decrease in number gradually from day 3 to 18. Decreasing the number of small follicle might have resulted from the decrease in FSH-surge as observed in cattle (Adams *et al.*, 1992) and/or due to the suppressive effect of DF which usually dominate on d4 or d5 of the cycle (Ginther *et al.*, 1998).

Follicles between 5 and 8 mm in diameter fluctuated in number from stage to stage. They were low at the end of cycle (between d18 and d21), increase significantly at the beginning of the cycle (between d1-d4), then decreased slightly between d5 and d10 to increase again between d11 and d17. The large number of those follicles seen between d1 and d4 of the cycle might have resulted from growth of a large pool of small follicles present earlier in the cycle. The fluctuation in development of this class of follicles suggests that, they might have grown in a wave pattern, one wave between d1 and 4 and the other between d11 and d17. In cattle, after emergence of small follicles on d1, they develop together till divergence and dominance of a DF on d4 or d5, then the DF suppresses the growth of other follicles in the same wave between d5 and d10, when another wave start to develop (Ginther *et al.*, 1996).

Whenever the suggestion of the wave pattern of follicular growth is true, then why the small follicles (<5mm) did not increase between d11 and 17, as their increasing between d1 and d4?. This might be due to 1) As will be seen, not all the observed animals in the current study showed a second wave of follicular growth, but some animals showed only one wave at the begin of the cycle. 2) Ali (2000) recorded in Fresian heifers that small follicles (<5mm) did not fluctuate in number allover the estrous cycle. 3) Matton *et al.* (1981) observed also in cattle that, small follicles decreased from begin to the end of the cycle. 4) In cattle (Savio *et al.*, 1988 and Sirios and Fortune, 1988) and buffalo (Manik *et al.*, 1994 and Taneja *et al.*, 1996) the first follicular wave begin on d1 and start to decrease only after the 4th day. So this stage of cycle (d1-d4) showed only increasing in the number of follicles. On the other hand, the second follicular wave appears between d9 and d11, then start to decrease 4 days latter, meaning that, the third stage of cycle (d11-d17) show both increase and decrease in the number of small follicles.

The number of follicles between 9 and 12 mm in diameter were higher in number at the mid- (between d5 and d17) than at beginning (d1-d4) or at the end (d18-d21) of the cycle. This might have resulted from development of the medium sized follicles observed in high number between d1 and d4.

Follicles more than 12 mm in diameter increased steadily in number from beginning to the end of estrous cycle, which is in agreement with that recorded in cattle (Matton *et al.*, 1981 and Kahn, 1989).

The incidence of presence of follicle more than 8 mm in diameter increased steadily from 25% at the beginning of the cycle to 100% at the end. In cattle, at least one follicle within the size from 10 to 20 mm was usually present from d4 or d5 of the estrous cycle on to ovulation (Rajakoski, 1960; Ireland *et al.*, 1979; Matton *et al.*, 1981 and Kahn, 1989). Large follicles could be palpated during d9 to d13 of the estrous cycle in 65% of post-pubertal buffalo heifers (Singh *et al.*, 1984). However, presence of large follicle between d1 and d4 is unusual, which might be originated from an anovulatory follicle from the previous cycle, as it was recorded in cattle (Knopf *et al.*, 1989).

DF between d5 and d10 was located on the left more than on the right ovary, while between d11 and d21 it was found equally distributed between right and left one. This means that, DF may have changed its position from one side to the other during the different stage of cycle. In other words, DF of the former stage (d5-d10) is not the same one of the latter one (d11-d21), suggesting the development of new DF.

During early diestrus, the DF was in the most cases functionally active (E2-dominant,) and non-atretic. However, half of the DF in second stage (late diestrus) became functionally inactive (P4-dominant) and atretic. Towards the days of proestrus and estrus, DF becomes in all cases functionally active and non-atretic. This is in agreement with the findings of other studies (Danell, 1987 and Price *et al.*, 1995). This turnover in the hormonal and histological status of the DF supports the hypothesis of wave pattern of follicular growth.

About one third of animals during diestrus showed two large follicles, which is in accord with other reports in cattle (Ireland *et al.*, 1979 and Kahn, 1989). Histopathological examination revealed that both follicles were atretic in 22.2%, while in the majority of cases (77.7%) one follicle was atretic and the other was non-atretic. The larger follicle was atretic in half of the cases. In cattle, the ratio of atresia between d13 and 18 was more significant than that at other stages (Rajakoski, 1960). Also, in Surti buffalo heifer during midcycle, 6/11 of the largest follicle were normal and 5/11 were atretic (Danell, 1987). These observations support the opinion of follicular wave growth, especially when the larger follicle was atretic, while the second larger one was non-atretic. The difference in the atretic status of both large follicles suggest that, they have originated from different waves, one of the current wave (non-atretic) and the other from the preceding wave (atretic). Singh *et al.* (2000) observed also two follicular growth waves during the cycle in buffalo heifers of the Murrah breed.

Furthermore, hormonal analysis of the two large follicles during diestrus revealed that, in most cases (80%) both follicles were different in the hormonal status, where one was active (E_2-dominant) and the other was inactive (P_4-dominant). This also supports our suggestion of the wave pattern of follicular growth. Staigmiller and England (1982) observed that high concentration of E_2 was found only in large follicle, but not all large follicle had high concentration. Cows with more than a single large follicle did not have more than one containing high concentration of E_2. Price *et al.*, 1995) also reported in cattle that, E_2 concentration was lower in static than in growing follicle and much lower in regressing one. Moreover, E_2-content was lower in the histologically atretic than in nonatretic and early atretic follicles. P_4 was higher in regressing than in growing follicles.

About 15% and 20% of animals during early and late diestrus, respectively, did not show follicles larger than 8 mm, while all the observed animals showed a large follicle during following stage. This suggested that, these buffalo-cows showed only one wave in their cycle. Savio *et al.* (1988) reported in 1/13 heifers an estrous cycle with only one wave. DF was observed on d3, reached maximum diameter of 16 mm on d7, then decreased in diameter on d8 and d9, to increase again and reach maximum diameter of 20 mm on d20. Two pattern of follicular growth was observed in 5 Murrah buffalo-cow examined with ultrasound for 7 cycles (Taneja *et al.*, 1996). Single wave was observed in 3/7 and 2 waves in 4/7 cycles. However, Manik *et al.* (1994) revealed a predominance of two wave follicular activity (5/6) in 6 Murrah buffalo. Three waves cycle was recorded in only one case.

According to the present observations using a morpho-histo-endocrinological model, it could be concluded that, Egyptian buffaloes posses two patterns of follicular growth. While some cows showed only one wave of follicular growth, the majority showed two waves-like patterns. Such pattern of follicular growth should be considered on the design of estrous synchronization and superovulation programs.

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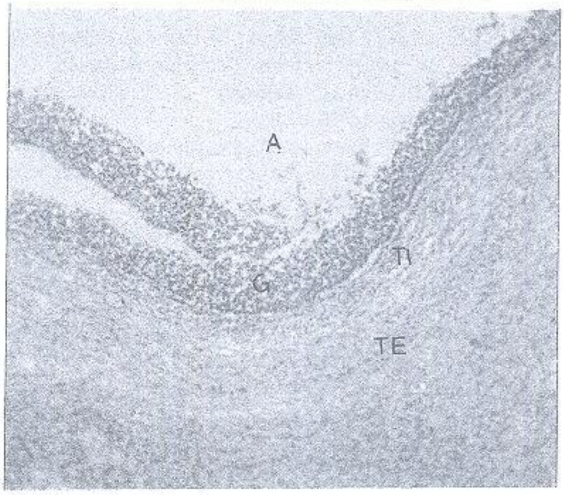

Fig 1: Section of wall of a normal dominant follicle, Antrum (A), granulosa (G), theca interna (TI) and theca externa (TE). H & E, 10x25.

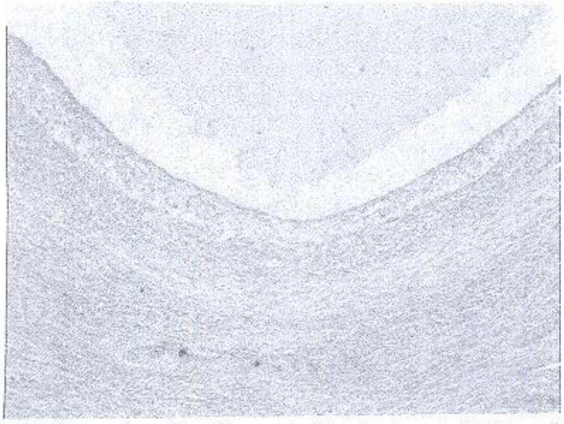

Fig 2: Section of wall of an atretic dominant follicle showing disappearance of the cumulus and granulosa layer that are replaced by connective tissue. H & E, 10x25.