

Effects of Exogenous GnRH, Calcium and Bromocriptine on Vaginal Smear Cytology and Ovarian Activity of New-Zealand Rabbits

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Abstract: This study aimed at displaying the histo-morphological changes in the vaginal cells and ovaries in response to GnRH, calcium, or bromocriptine. A total of 40 lactating NZW does were assigned into four comparable groups three of which were administered by (GnRH-G), calcium (Cal-G) and bromocriptine, (Br-G) while the fourth was given saline as control. Changes occurring in the vaginal cells and ovaries were demonstrated. Linear regression analysis revealed that the coefficient of determination (R^2) between parabasal cells, intermediate, superficial and anuclear cells and follicular population was 0.92, 0.42, 0.39 and 0.35, respectively. The total ovarian follicle number was significantly lower in Br-G and GnRH-G at two and four hours post-treatment, respectively, when compared to that in control. Ovarian interstitial tissue showed hypertrophy and hyperplasia in GnRH-G and was associated with higher rate of atresia and pyknosis in Cal-G and Br-G. From these results, it can be concluded that exfoliated parabasal cells in the vaginal smear might be helpful to express the ovarian function in does; prolactin suppression up to eight hours resumes the subsequent follicular growth and hence improve fertility. Calcium administration might have a synergistic action with pituitary gonadotropins at the ovarian level. Moreover, calcium and bromocriptine administration have the ability to increase the follicular population in does, even though different mode of action.

Key words: Bromocriptine • Calcium • Does • GnRH • Ovary • Vaginal Smear

INTRODUCTION

Female rabbits are classified as induced or reflex ovulators where ovulation is incorporated with mating, an evidence which differ from that in cattle [1]. Unlike most other mammals, the formation, activation and development of ovarian follicles seem to occur entirely post-natally in rabbits, where the primordial follicle assembly presumably is completed between 2 and 4 weeks of age [2]. With the onset of puberty, follicles reach the ovulatory status in association with the sexual behavior. It has been indicated that follicles >1.8 mm diameter were present only in the receptive females [3], in addition to pre-ovulatory follicles exceed of > 800-900 μ m in diameter or >1.5 mm-> 2 mm in diameter [4, 5].

In rabbits, ovulation can be induced by the exogenous administration of hormones like GnRH [1], which increases the synthesis and secretion of FSH and

LH enough to promote the development of ovarian follicles [6] and to induce the ovulation in does.

Ovarian smooth muscle activity has been considered to be one of several factors responsible for mammalian ovulation. It has been reported that the ovarian smooth muscle contractile activity is functioning with the ovulatory process [7]. Oral administration of calcium to adult non-pregnant female rabbits was found to cause a significant increase of serum levels of estradiol and follicle stimulating hormone [8]. So far, there is no detailed study signified the effect (s) of calcium on the ovarian function and folliculogenesis in rabbits.

It has been reported that the postpartum anestrous period is longer in suckled or intensively milked animals [9]. Suckling tends to suppress growth of follicles [10] and hence blocks ovulation [11]. Partial or restricted suckling and early or temporary weaning (also called strategic weaning) can reduce the postpartum anestrous period and

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thus improve pregnancy rates [12]. The difference in fertility of lactating does is systematic, being 10-20% in favor of non-lactating does [13]. Such difference is likely associated with the depressing effect of lactation on the ovulation ability which is 68% in lactating and 91.5% in non-lactating [14]. Prolactin (PRL) secreted from the anterior pituitary gland well known for its effects on the breast, promoting mammary growth and lactation, in addition to its necessity for the maintenance of the corpus luteum [15]. Prolactin deprivation by bromocriptine 2-bromo- α -ergocryptine-methane-sulphonate has been suggested to have no effect on the number of follicles or on the amount of LH receptor mRNA particularly on day 11 of lactation [16].

The present study was designed to define the correlation between vaginal epithelial cells and ovarian activity in rabbit does as well as to determine the rate of ovarian response to GnRH, calcium, or bromocriptine (prolactin deprivation) administration in the lactating does.

MATERIALS AND METHODS

Animals: A total of 40 lactating multiparous New Zealand white rabbits, 3.0-3.5 kg in weight, were used in the present study. All does were fed on a commercial pelleted diet (16.3% crude protein, 13.2% crude fiber and 2.5% fat) and kept on the same system of management as previously described [17].

Experimental Design: Does were allotted equally to four experimental treatments (n=10 per group). Each group was adopted one treatment two hours before introduction of females for natural mating. Group 1 (GnRH-G); injected s/c by 0.2 ml GnRH analogue; buserelin acetate (Receptal®, 4.2 μ g/ml, Intervet/Schering-Plough Animal Health). Group 2 (Cal-G) administered orally 100 mg/kg B.Wt. calcium carbonate (Calcichew®, 500 mg/tablet, Shire Pharma. Co., Egypt) dissolved in 8ml of saline [15]. Group 3 (Br-G) also administered orally 5 mg bromocriptine mesilate (Parlodel®, 5 mg/tablet, Novartis Pharma Co., Egypt) dissolved in 8 ml of saline [18]. Group 4 or control group was received an equivalent volume of saline.

Vaginal Smear Collection and Preparation: Exfoliated vaginal cells were collected aseptically after treatment and at two hours interval up to eight hours, by swapping the vaginal wall using a 5 cm sterile cotton swab which rolled on a glass microscope slide, air dried and stained by the diluted (1:10, V/V) Giemsa

stain (Sigma-Aldrich, Germany) as described previously [19] and examined under the light microscope ($\times 100$). According to Tsiligianni *et al.* [19], the predominant cell types in the vaginal smear were classified into parabasal (round or nearly round cells, have a high nuclear to cytoplasmic ratio), intermediate (vary in size and shape, but typically have a diameter 2-3 fold of parabasal cells), superficial (flat polygonal cells with pyknotic nucleus) and anuclear (cornified polygonal cells without nuclei and are often seen in large sheets or strings). At least a total of 200 cells per vaginal smear were counted and the ratio of different cells types was calculated and expressed in percentage to the total number of epithelial cells in each smear. Accordingly, the changes in parabasal cells in rabbit vaginal smear might be used to predict and/or calculate the total follicle population on the ovary according to the linear regression analysis equation $Y = 3.34 \times -1.68$, where Y is total follicle number and \times is the number of parabasal cells.

Ovarian Histological Preparation and Evaluation:

At the end of the experimental period, four does from each group were sacrificed, ovarian samples were collected, fixed in 10% formaldehyde, before being dehydrated in ethanol (70, 90 and 100%) and embedded in paraffin wax. Ovarian slides were prepared for the histological examination after staining by haematoxylin and eosin (H and E). The diameter (μ m) of follicles was computed using ImageJ software, a publicly available image analysis program. Follicles which contain successively more cuboidal granulosa cells in layers around the oocyte were classified into primary, secondary or tertiary as described by Zitni *et al.* [20]. Follicles were subdivided according to their size into small primary follicles (SPF), large primary follicles (LPF), small secondary follicles (SSF) large secondary follicles (LSF) and tertiary follicles (TF) which have $< 200 \mu$ m, 200-450 μ m, 450-700 μ m, 700-1000 μ m and $> 1000 \mu$ m in diameter, respectively [21]. Follicles were considered atretic if two or more pyknotic granulosa cells were found on the same section or if the oocyte completely degenerated [22].

Statistical Analysis: The obtained data were tabulated and statistically analyzed by using SPSS (Ver. 14) and graphed using GraphPad Prism (Ver. 5.0, San Diego, CA, USA). Results were expressed as means (\pm SEM) and statistically analyzed using analysis of variance and Duncan's Multiple Range Test. Pearson correlation analysis was run between the ovarian follicle population and epithelial cell types.

RESULTS

Correlation Between the Changes in Vaginal Smear Cells and Follicular Population: As shown in fig. (1), There was a close relationship ($R^2=0.92$) between parabasal cells and follicular population (fig. 1A). In the meantime, a lower magnitude of correlation between either the intermediate, superficial or anuclear cells and follicular number was recorded (0.42, 0.39 and 0.35, respectively) (Fig. 1B, 1C and 1D).

Changes in Follicles Number in Relation to Time after Various Treatments: As indicated from fig. (2), there was a significant variation in the calculated total follicle number (CTFN, (mean± S.E.)) in association with the time elapsed after different treatments. CTFN was significantly ($P<0.05$) lower in Br-G than that in control (125 ± 13 vs. 224 ± 14) at two hours and in GnRH group at four hours as compared with control group (148 ± 24 vs. 247 ± 21). At eight hours, CTFN in Br-G was significantly higher than in control group (210 ± 13 vs. 111 ± 37).

Ovarian Morphological Changes after GnRH, Calcium and Bromocriptine Treatments: As revealed from fig. (3), follicles in the GnRH-G showed multiple layers of intact granulosa cells surrounding the oocyte (Fig. 3A),

while there was cytoplasmic vacuolations and pyknosis and absence of oocytes in many follicles in Cal-G and Br-G (Fig. 3B and 3C, respectively).

The distribution of follicles in percentages, follicular diameters (healthy and atretic) in different treated does were presented in table 1. The total number of follicle and number of primary, secondary and tertiary follicles were presented in figure 4. The overall mean follicle number was significantly higher in Cal-G than in GnRH and control groups.

Small Primary Follicles (SPF): The number of healthy small primary follicles ($< 200 \mu\text{m}$) in ovaries of treated does was characterized by a marked ($P<0.05$) increase in Br-G than GnRH group. However, the mean diameter of healthy small follicle population insignificantly differed between various groups. The number of atretic SPF was significantly ($P<0.05$) higher in Cal-G than GnRH and control groups. The mean diameter of atretic follicles was significantly larger in Cal-G than that recorded in GnRH-G (154.26 ± 1.74 vs. $120.14\pm 12.51 \mu\text{m}$).

Large Primary Follicles (LPF): The healthy and atretic large primary follicles ($200-450 \mu\text{m}$) population were significantly ($P<0.05$) higher in Cal-G than that in GnRH and control groups.

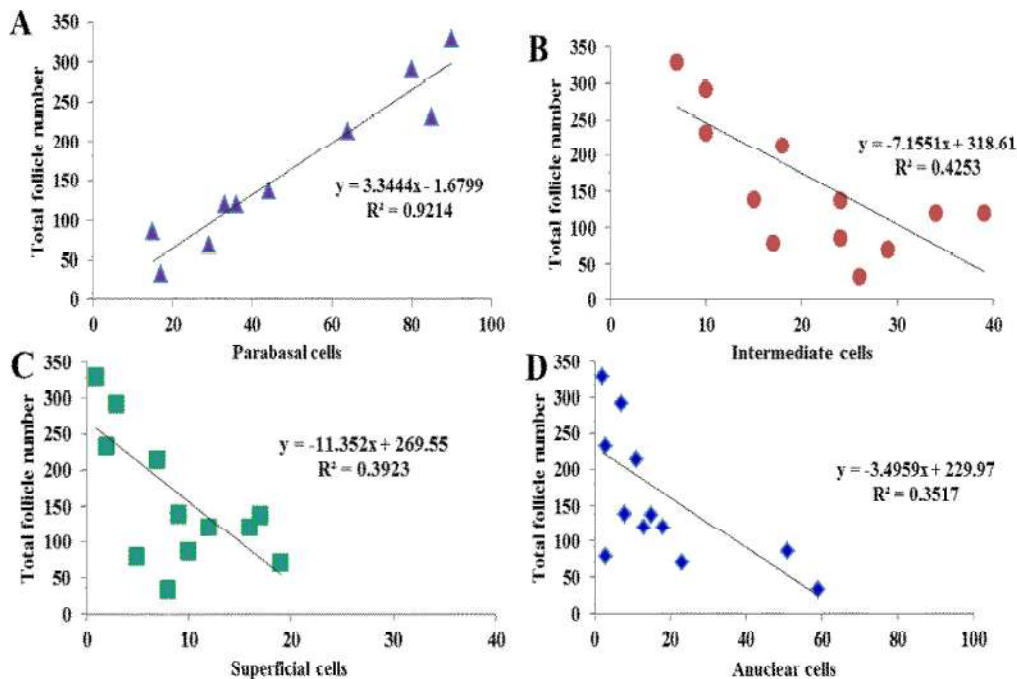


Fig. 1: Correlation between vaginal exfoliated cells and follicular population in rabbits. Note that while parabasal cells and total follicle numbers were closely correlated (A). While the intermediate, superficial and anucleated cells were negatively correlated (B, C and D, respectively).

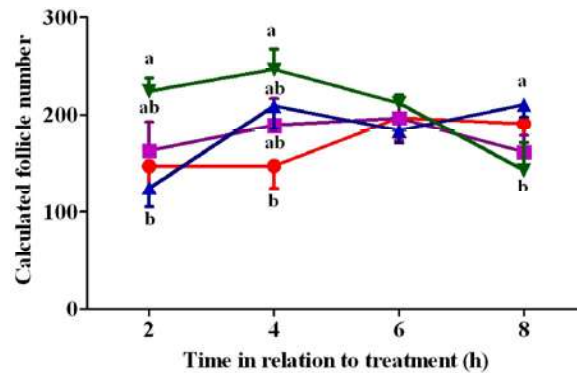


Fig. 2: Changes in calculated follicular number in relation to time in GnRH (●), Calcium (■), bromocriptine (▲) and control (▼) groups. Values with different letters at the same time point were significantly different at $P < 0.05$.

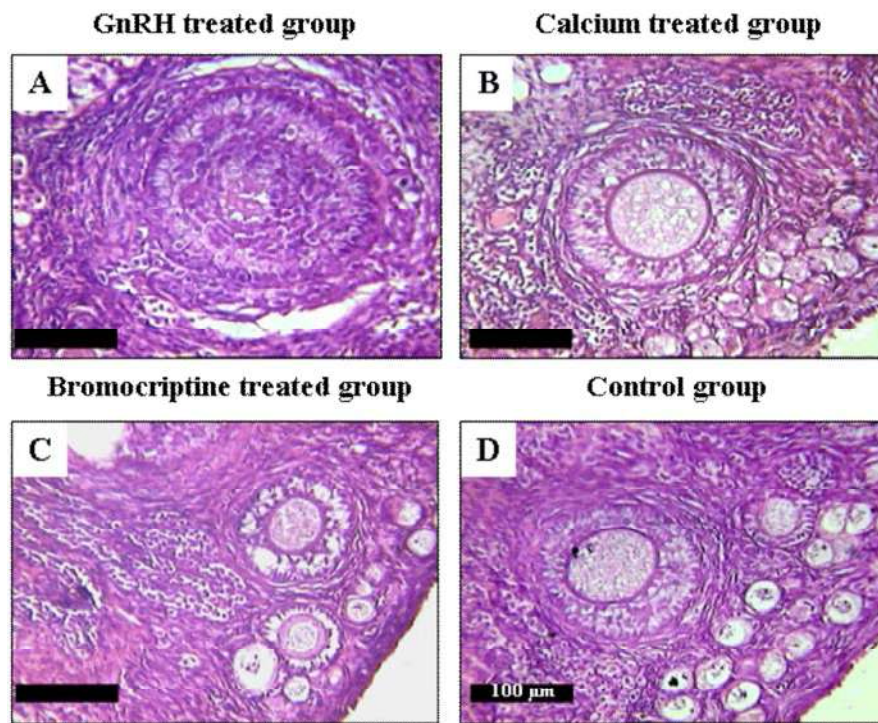


Fig. 3: Morphological characteristics of growing follicles on the ovaries of GnRH (A), Calcium (B), bromocriptine (C) and control (D) groups. Note the high proliferative effect of GnRH on granulosa cells. In the meantime, conspicuous cytoplasmic vacuolations and pyknosis in follicles of calcium and bromocriptine groups. All pictures were photographed at the same magnification (H and E $\times 40$, Scale bar=100 μm).

Small Secondary Follicles (SSF): The number of total healthy small secondary follicles (450-700 μm) did not differ significantly between various treated groups. The number of atretic SSF was significantly ($P < 0.05$) higher in Br-G than GnRH and control groups.

Large Secondary Follicles (LSF): Although the population of LSF (700-1000 μm) was not significantly different between treated groups and control, there was difficulty to find healthy follicles in bromocriptine treated

group. The number of atretic LSF was significantly higher in calcium group than GnRH group but the diameter of observed LSF slightly differed between treated groups when compared to control.

Tertiary Follicles (TF): By the end of experiment, examined ovaries of GnRH, Br-G and control groups were interestingly had very few healthy large tertiary follicles ($> 1000 \mu\text{m}$); meanwhile atretic follicles were visible on the ovary of GnRH and control groups.

Table 1: Effect of treatment with GnRH, calcium and bromocriptine on mean diameter and percentage of ovarian follicular populations in lactating rabbit does

Follicle categories	Follicle status	Animal groups			
		GnRH	Calcium	Bromocriptine	Control
Small primary follicles (<200 μm)	Healthy	127.03±9.80	127.56±0.75	122.00±4.24	125.68±3.83
	Atretic	120.14±12.51 ^b	154.26±1.74 ^a	134.38±1.70 ^{ab}	134.65±6.59 ^{ab}
	%	51±5 ^{ab}	42±5 ^b	56±4 ^a	58±4 ^a
Large primary follicles (200-450 μm)	Healthy	327.07±13.20	338.01±12.01	292.73±21.18	320.05±10.03
	Atretic	323.52±7.71	323.84±0.82	321.88±10.78	279.65±30.96
	%	32±4 ^{ab}	42±6 ^a	29±2 ^{ab}	26±3 ^b
Small secondary follicles (450-700 μm)	Healthy	510.12±15.98	509.73±32.13	558.21±4.81	564.37±20.08
	Atretic	562.24±2.56	544.79±14.14	578.05±23.02	578.88±24.35
	%	10±2	12±4	13±2	8±1
Large secondary follicle (700-1000 μm)	Healthy	766.92	-	-	714.06
	Atretic	766.78±23.00	741.73±2.61	748.01±23.21	808.00±57.05
	%	4±2	4±1	2±1	4±2
Large tertiary follicles (> 1000 μm)	Healthy	766.92	-	-	-
	Atretic	1089.99±24.34	-	-	1080.51±28.61
	%	3±2	0	0	4±1

Values (mean ±SE, n=4) within the same column row with different letters were significantly different at P< 0.05. % indicated relation between follicle population (healthy and atretic) in relation to total ovarian follicular population per doe ovaries.

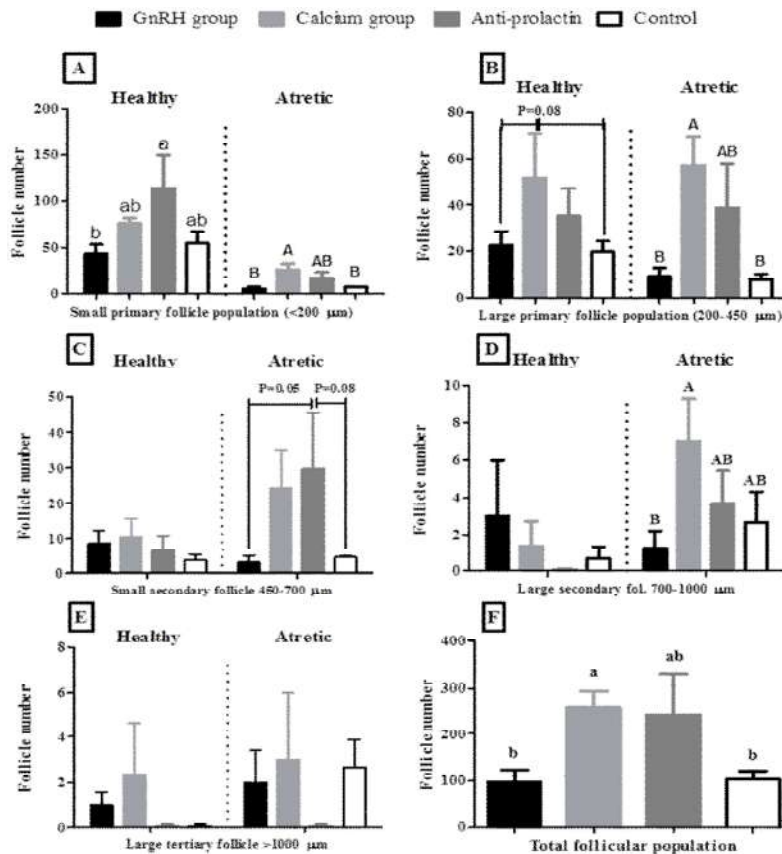


Fig. 4: Changes in ovarian follicular populations in GnRH (■), Calcium (■), bromocriptine (■) and control (□) groups. Ovaries of slaughter rabbits (n=4 pairs/group) were processed for histological evaluation at the end of experimental period (8 hours). Follicles were categorized into small primary follicles, = 200 μm in diameter (A); large primary follicles, 200-450 μm in diameter (B); small secondary follicles, 450-700 μm in diameter (C); large secondary follicles, 700-1000 μm in diameter (D); tertiary follicles, > 1000 μm in diameter (E) according to their size. Values (mean ±SE) with different letters were significantly different at P<0.05.

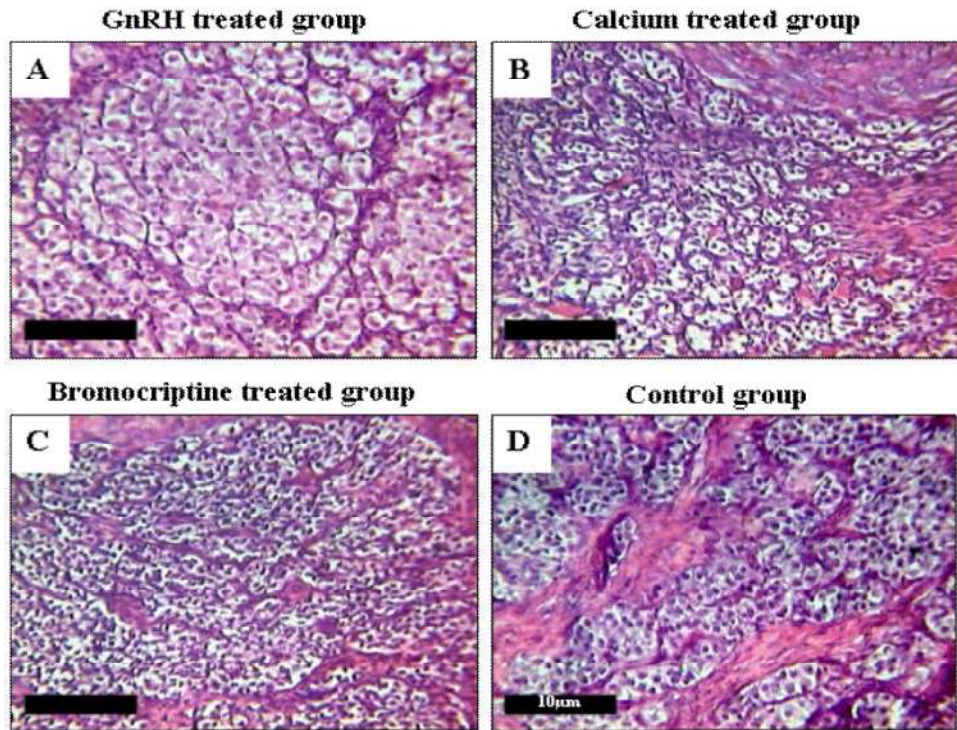


Fig. 5: Morphological characteristics of interstitial cells in the ovaries of GnRH (A), Calcium (B), bromocriptine (C) and control (D) groups. Note the hypertrophy and hyperplasia of interstitial cells in GnRH group. In the meantime, hyperplasia and high rate of pyknosis in calcium and bromocriptine groups. All pictures were photographed at the same magnification (H and E $\times 100$, Scale bar=10 μm).

Ovarian Interstitial Cells Characteristics: As shown from fig. (5), GnRH-G showed hypertrophy and hyperplasia of ovarian interstitial cells when compared to control group. However, the Cal-G and Br-G revealed that in addition to hyperplasia of the ovarian interstitial cells, there was high rate of atresia and pyknosis in Cal-G and Br-G.

DISCUSSION

The value of the vaginal smear method for monitoring the ovarian functions depends upon the response of the vaginal epithelium to the hormones of the ovary [19]. The current study verified a close linear relationship ($R^2=0.92$) between parabasal cells and total follicular number in treated does, a finding which can be used as an indicator of for the ovarian status in rabbits. This finding came to support the close relationship between the exfoliated vaginal cytology and the effect of the ovarian hormones, particularly estrogen/progesterone ratio in rat [23] or its relation with the ovarian yielding capacity of corpora lutea, oocytes and number of normal zygote in rabbits [19].

In the present study, there was a significant decrease in the CTFN for Br-G and GnRH-G at two and four hours, respectively, followed by an increased in Br-G only at eight hours when compared to control. The lower follicular growth in GnRH and Br-G perhaps was due to high progesterone production from interstitial cells in mouse [24]. Prolactin increases tuberoinfundibular dopamine turnover, which has been demonstrated to suppress hypothalamic GnRH [25] and therefore prolactin deprivation by bromocriptine probably results in stimulation of GnRH, the importance of which in regulating the activity of interstitial cells is well established [26].

By the end of the experiment, results showed that follicular populations in GnRH group did not vary from control and this perhaps was due to the decreasing GnRH levels and consequently its lower effect on follicle growth. It has been reported in human [14], following the highest concentration in GnRH treatment 2 h post-mating, LH concentration showed significant decrease 3 h post-mating and FSH concentration significantly decreased 4 h post-mating. Although, the diameter of atretic LPF seemed to be larger, healthy SSF was slightly smaller in

GnRH than control group. This finding might be attributed to the pressure atrophy of hypertrophied interstitial cells on the developing follicles under effect of GnRH stimulation. Singh and Krishna [27] showed that pharmacological dose of GnRH showed inhibitory effect on ovarian follicular development and steroidogenesis in mice, but re-injection of GnRH agonist significantly increased the diameter of primary follicles when compared to control in does [16].

Looking through the prominent changes in the ovarian structures for the Cal-G, the role of calcium has been emphasized as a ubiquitous intracellular signaling molecule responsible for initiating specific events associated with regulation of folliculogenesis and might be involved in gonadotrophic hormone action at the ovarian level [28]. The inhibitory and stimulatory effects of GnRH are mediated through the binding of the peptide to high-affinity receptors in granulosa and thecal cells, based on calcium mobilization and probably operates through stimulation of phospholipid turnover and activation of protein kinase C [29]. It has been mentioned that, the oral administration of calcium to adult non-pregnant female rabbits causes a significant increase in serum levels of estradiol and follicle stimulating hormone [15]. FSH has been found to induce transient calcium elevations, whereas hCG causes a prolonged elevation and marked oscillations in calcium [30].

In the present study, bromocriptine treated does were generally had a higher total number of follicles than GnRH and control groups, though it was significantly proven. Similarly, it was found that bromocriptine treatment had no effect on the number of follicles in rabbit ovary, but perhaps on the activity of follicles during lactation [16], indicating that prolactin may exert an antifertility effect at the ovarian level in ewes [31]. A significant higher rate of atretic SPF than that in GnRH group, might indicate that prolactin may influence the viability of gonadotrophin-responsive follicles, a finding which support the potential use of bromocriptine as co-treatment in follicular stimulating protocols. It has been recorded that, the high concentrations of prolactin observed during lactation coincide with a reduction in gonadotrophin secretion and down-regulation of the synthesis of the LH receptor in ovarian follicles of lactating rabbits [16]. Bromocriptine acts by allowing FSH to rise above threshold requirements for follicular stimulation [32]. However, the significant higher rate of atretic SSF in Br-G than GnRH might be attributed to the lowered pituitary LH secretion in association with the latent effect of prolactin in

lactating does, too high to bypass the single dose treatment of bromocriptine. It has been found that bromocriptine administration markedly decreases prolactin secretion, LH pulse frequency, but has no effect on FSH concentration [31, 33], also the repeated bromocriptine administration was indicated to delay the decrease of follicular estrogen production and the occurrence of structural signs of atresia in rat [34].

From this study we could deduced that parabasal cells may reflect total follicular number in does. Calcium and bromocriptine administration are beneficial to increase the follicular population in does.

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