Interrelationships Between Follicular Size, Estradiol-17β, Progesterone and Testosterone Concentrations in Individual Buffalo Ovarian Follicles

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ABSTRACT : This study was undertaken to measure the concentrations of estradiol-17β, progesterone and testosterone, and to study their relationship with each other and with follicular size in individual buffalo ovarian follicles categorized as small (4 to 5 mm diameter), medium (6 to 9 mm diameter) and large ($\geq$ 10 mm diameter). Steroid hormone concentrations varied markedly within follicles of each size category. Estradiol-17β concentrations (pmol/ml) were positively related to follicular diameter ($R = 0.34$, $n = 308$, $p < 0.001$) and were significantly higher ($p < 0.001$) in large (118.46 $\pm$ 30.25), compared to those in medium follicles (50.32 $\pm$ 8.29) which, in turn were significantly higher ($p < 0.001$) than those in small follicles (19.70 $\pm$ 5.57). Progesterone and testosterone concentrations (pmol/ml) were not related to follicular diameter and were not different among small (330.99 $\pm$ 27.32 and 17.68 $\pm$ 2.44, respectively), medium (384.84 $\pm$ 26.20 and 36.47 $\pm$ 4.55, respectively) and large follicles (253.25 $\pm$ 32.23 and 22.57 $\pm$ 4.48, respectively). Estradiol-17β and progesterone concentrations were positively related ($R = 0.39$, $n = 47$, $p < 0.01$) in small, unrelated in medium and negatively related in large follicles ($R = -0.59$, $n = 23$, $p < 0.01$). There was no relationship between estradiol-17β and testosterone concentrations in follicles of all the three size categories. Progesterone and testosterone concentrations were positively related in large follicles ($R = 0.57$, $n = 18$, $p < 0.02$). There was no relationship between the two hormones in small and medium sized follicles. When the follicles with estradiol-17β/progesterone molar ratios of > 1.00 were considered non-atretic, and the rest at different stages of atresia, 197/208 (95%) follicles were found to be atretic.

(Key Words: Buffalo, Estradiol-17β, Follicles, Progesterone, Testosterone)

INTRODUCTION

Follicular fluid is a complex mixture of restricted components of serum and follicular-synthesized secretions. It provides the means by which the cells of avascular granulosa are exposed to environments different from serum and specific for a follicle.

Alterations in the endocrine characteristics of the follicular fluid play a vital physiological role in follicular growth and development, steroidogenesis, ovum maturation and preparation of the follicle for subsequent corpus luteum function (McNatty, 1978). The hormonal milieu within each follicle is characteristic of that follicle and has been reported to depend upon the follicular size, stage of estrous cycle and degree of atresia in cattle (Kruip and Dieleman, 1986; Wise, 1987). The information available on the concentrations of estradiol-17β, progesterone and testosterone in buffalo ovarian follicular fluid (Kulkarni et al., 1994; Parmar and Mehta, 1994; Nigam et al., 1995) suffers from two limitations 1) the three hormones were estimated in different and not the same follicles and 2) the hormone estimations in small sized follicles were conducted in pools of follicular fluid. There is no information available on the interrelationships between different steroid hormones in individual buffalo ovarian follicles.

We have earlier developed a direct radioimmunoassay (RIA) for estradiol-17β (Palta et al., 1996) and a highly sensitive enzymeimmunoassay (EIA) for progesterone (Prakash et al., 1997), each of which requires only 5 μl of follicular fluid. This enabled us to measure the concentrations of estradiol-17β, progesterone and testosterone in individual follicles of all size categories. The present study was undertaken to measure the concentrations of these three hormones in individual buffalo ovarian follicles and to study their relationships with each other and with follicular size.

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MATERIALS AND METHODS

Collection of follicular fluid

Ovaries were obtained from apparently healthy buffaloes from abattoir and brought over to the laboratory at 4℃ in isotonic saline medium containing penicillin (200 I.U/ml) and streptomycin (200 μg/ml). The follicular size was measured with a real time B-mode ultrasound machine (Tokyo-Keiki LS-1000) with a linear array 5 MHz transducer as described earlier (Palta et al., 1996). The follicles were classified as small (3-5 mm diameter), medium (6-9 mm diameter) and large ($\geq$ 10 mm diameter). Follicular fluid (bFF) was harvested from individual follicles with a 50/100/250 μl Hamilton syringe and diluted 1 : 10 in isotonic saline.

Cellular debris was removed by centrifugation at 4,000 xg for 15 min at 4℃. These prediuted bFF samples were stored at −20℃ until subsequent analysis for estradiol-17β, progesterone and testosterone.

Estimation of steroid hormones

Estradiol-17β was measured by a direct RIA described earlier (Palta et al., 1996). In brief, the reaction mixture consisted of duplicate bFF samples (20 to 50 μl of 1 : 10 pre-diluted bFF samples), 0.1 ml anti-estradiol-17β serum (1 : 70,000), 0.2 ml of phosphate buffer saline, 0.1% BSA, pH 7.2 and 0.1 ml (7,000 cpm) of 1, 2, 6, 7-^{3}H testosterone (Amersham International plc, U. K). The volume was made up to 400 μl and the tubes incubated at 4℃ overnight. The bound and free fractions were separated by addition of charcoal-dextran. The sensitivity of the assay was 0.4 ng/ml. The intra- and inter-assay coefficients of variation were < 13%.

For estimation of testosterone by RIA, duplicate bFF samples (50 or 100 μl of 1 : 10 pre-diluted samples) were placed in 12 × 100 mm test tubes and extracted with 2.0 ml diethyl ether for 1 min, as described earlier for estradiol-17β (Palta et al., 1996). The reaction mixture consisted of 0.1 ml anti-testosterone serum (1 : 20,000), 0.2 ml of phosphate buffer saline, 0.1% BSA, pH 7.2 and 0.1 ml (8,000 cpm) of 1, 2, 6, 7-^{3}H testosterone (Amersham International plc, U.K.). The rest of the procedure was similar to that for estradiol-17β as described earlier (Palta et al., 1996). The sensitivity of assay was 3.12 pg/tube and the intra- and inter-assay coefficients of variation were < 17%.

Progesterone was estimated by a highly sensitive enzymeimmunoassay (EIA) described earlier (Prakash et al., 1997). The heterologous EIA made use of antiprogesterone-7 α-carboxyethylthioether-BSA as the antibody and progesterone-6 β-hydroxyhemisuccinate-horse radish peroxidase as the enzyme label. The sensitivity of the assay was 0.4 pg/well and the intra- and inter-assay coefficients of variation were < 13%.

Statistical analyses

Follicular fiuid concentrations of all hormones were subjected to a logarithmic transformation prior to statistical analyses. Differences in mean hormone concentrations in follicles of different size categories were compared by least square analysis as described as Harvey (1976). The concentrations of estradiol-17β and progesterone were expressed in pmol/ml and molar ratios of estradiol-17β to progesterone concentrations were determined for each follicle. The correlations between, and regression analysis for follicular diameter, follicular fluid estradiol-17β, progesterone and testosterone concentrations were carried out as described by Snedecor and Cochran (1980).

RESULTS

Follicular fluid steroid concentrations vs follicular size

An ultrasound picture of a buffalo ovary containing follicles of various sizes is shown in figure 1. Mean follicular fluid steroid hormone concentrations in follicles

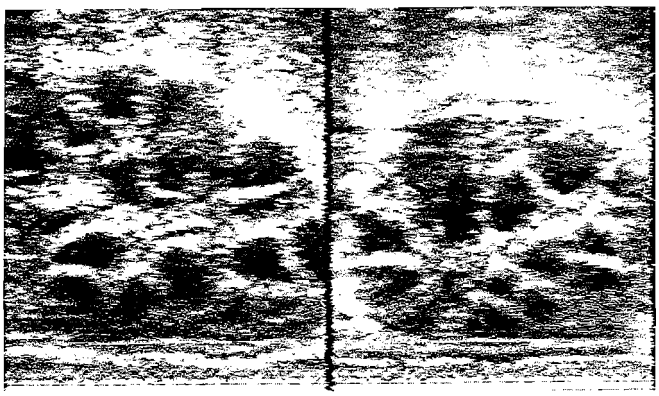


Figure 1. Sonograph of a buffalo ovary. Each black structure is a follicle.

of different size categories are presented in table 1. The concentrations of all the three hormones exhibited marked variations within each size category. Estradiol-17β concentrations (pmol/ml) ranged from 0.40 to 303.59 in small follicles, from 0.36 to 827.49 in medium sized follicles and from 0.73 to 1,038.00 in large follicles. Progesterone and testosterone concentrations (pmol/ml) ranged from 13.01 to 918.31 and 2.08 to 99.67, respectively, in small follicles, 13.67 to 1,677.86 and 0.62 to

Table 1. Concentrations of estradiol-17β, progesterone and testosterone (pmol/ml) in follicular fluid from individual buffalo ovarian follicles categorized according to follicular size

Hormone	Category	n	Concentration (pmol/ml)
Estradiol-17β	Small	83	19.70 ± 5.57[a]
	Medium	173	50.32 ± 8.29[b]
	Large	52	118.46 ± 30.25[c]
Progesterone	Small	77	330.99 ± 27.32
	Medium	169	384.84 ± 26.20
	Large	37	253.25 ± 32.23
Testosterone	Small	66	17.68 ± 2.44
	Medium	149	36.47 ± 4.55
	Large	31	22.57 ± 4.48

Small: 4-5 mm diameter; medium: 6-9 mm diameter; large: ≥ 10 mm diameter.
Different superscripts in the same column differ significantly ($p < 0.001$).

348.33, respectively, in medium sized follicles and from 25.43 to 792.68 and 0.54 to 98.74, respectively, in large follicles. Mean estradiol-17β concentrations were significantly higher ($p < 0.001$) in large follicles in comparison to those in medium sized follicles which, in turn were significantly higher ($p < 0.001$) than those in small follicles. The differences in follicular fluid mean progesterone and testosterone concentrations among follicles of different size categories were not significant. There was a significant positive relationship ($\ln y = 0.30 + 0.28 x$, $n = 308$, $R = 0.34$, $p < 0.001$) between estradiol-17β concentration (y) and follicular size (x), whereas progesterone and testosterone concentrations were not related to follicular size (figure 2).

Interrelationships between steroid hormones

There was a positive relationship ($\ln y = 5.00 + 0.29 \ln x$, $R = 0.39$, $n = 47$, $p < 0.01$) between estradiol-17β (x) and progesterone concentrations (y) in small follicles (figure 3A). There was no relationship between estradiol-17β and testosterone, and between progesterone and testosterone concentrations in small follicles (figures 3B and C). The three hormones were not related to each other in medium sized follicles (figure 4). Among large follicles there was a negative relationship ($\ln y = 6.80 - 0.36 \ln x$, $R = -0.59$, $n = 23$ $p < 0.01$) between estradiol-17β (x) and progesterone (y) concentrations (figure 5A). There was a positive relationship ($\ln y = 0.47 \ln x - 3.67$, $R = 0.57$, $n = 18$, $p < 0.02$) between progesterone

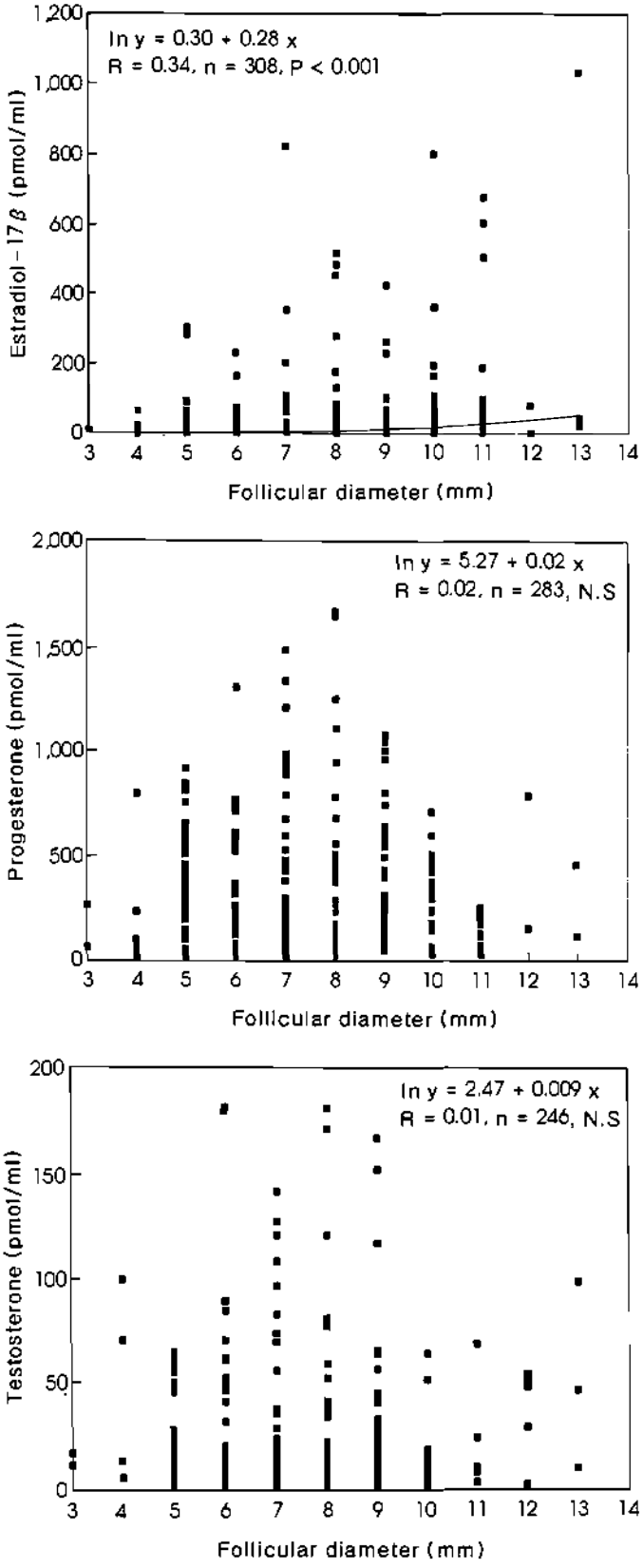
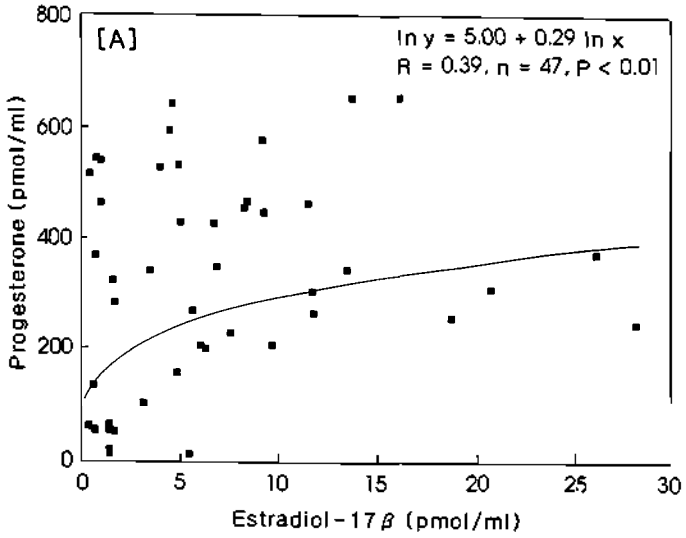
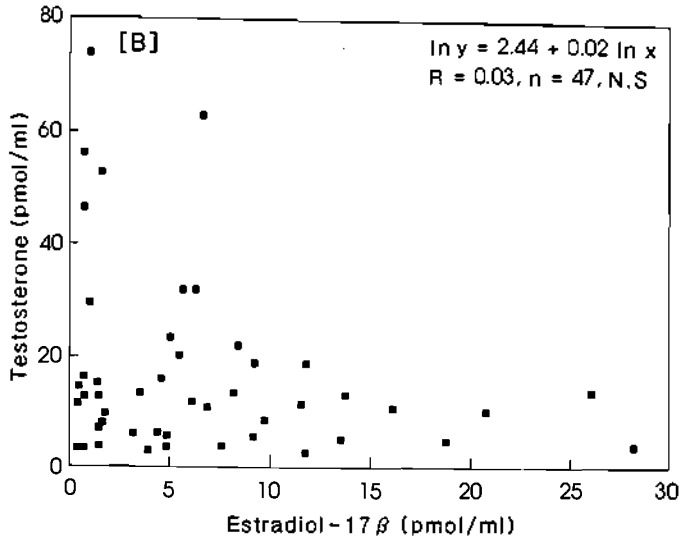


Figure 2. Relationship between estradiol-17β, progesterone and testosterone concentrations and follicular diameter in individual buffalo ovarian follicles.





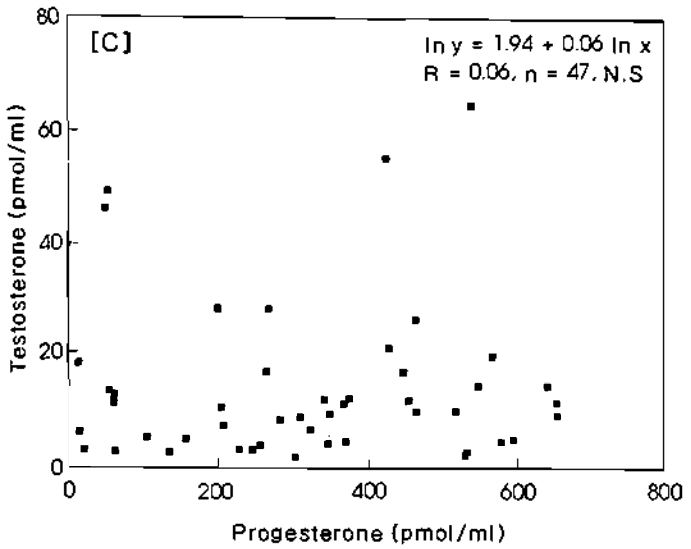
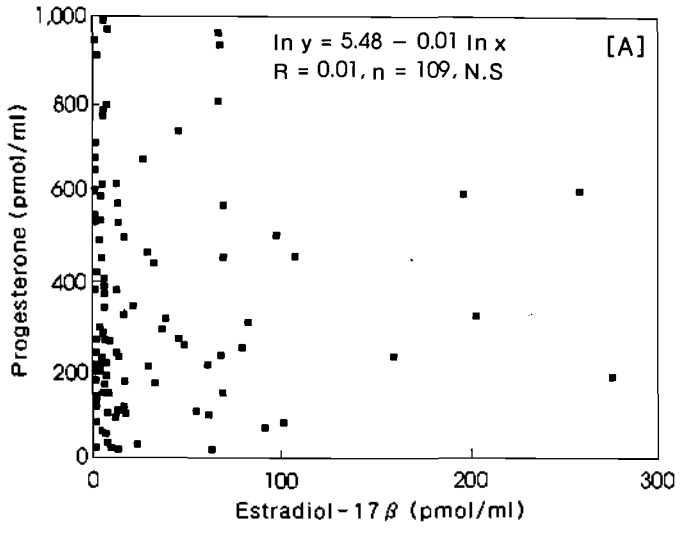
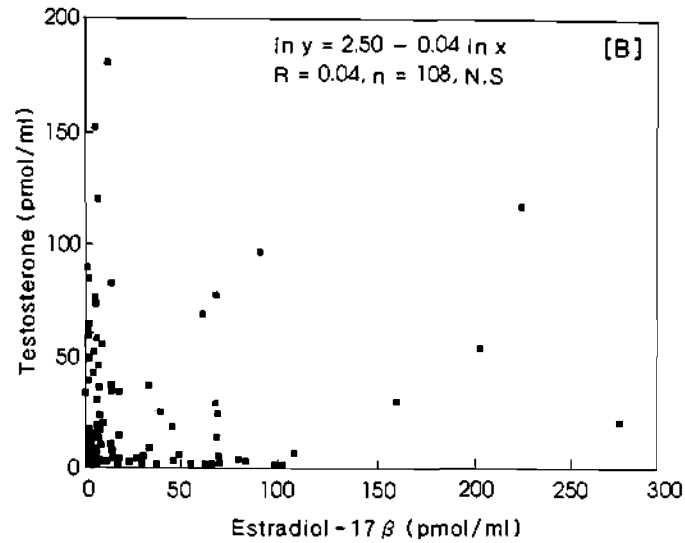


Figure 3. Interrelationship between concentrations of estradiol-17β, progesterone and testosterone in small-sized (3-5 mm diameter) buffalo ovarian follicles.





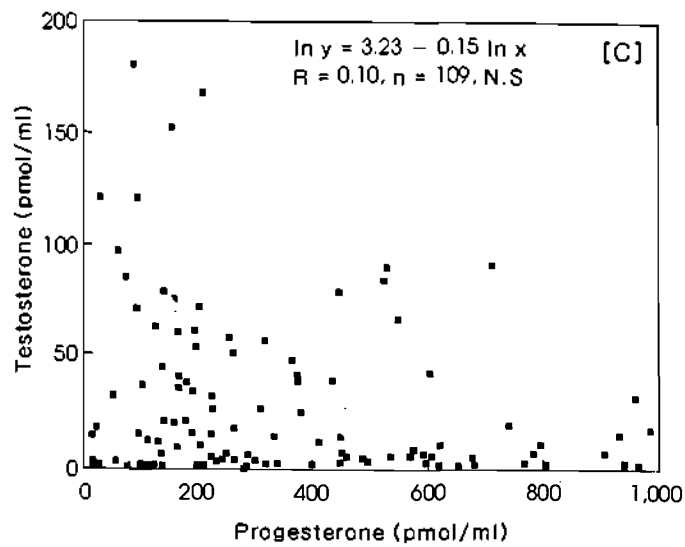


Figure 4. Interrelationship between concentrations of estradiol-17β, progesterone and testosterone in medium-sized (6-9 mm diameter) buffalo ovarian follicles.

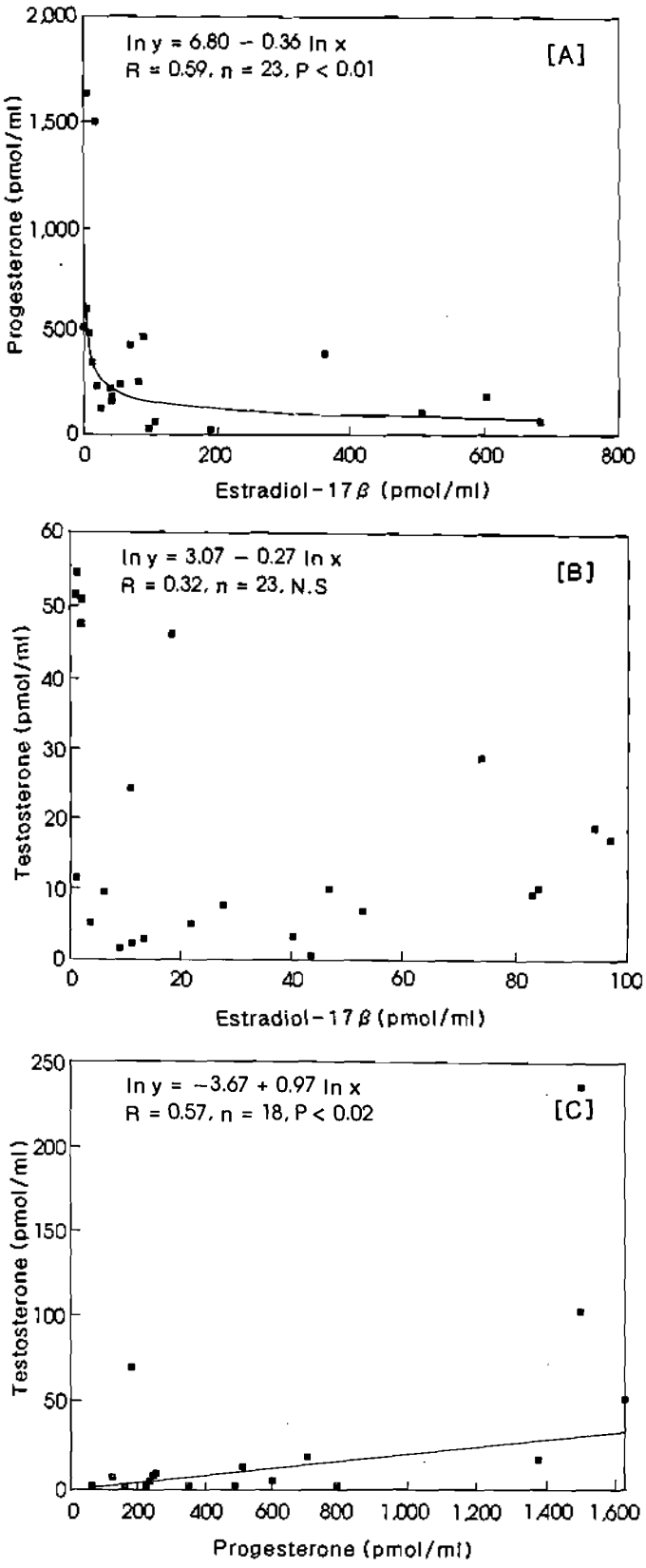


Figure 5. Interrelationship between concentrations of estradiol-17β, progesterone and testosterone in large-sized ($\geq$ 10 mm diameter) buffalo ovarian follicles.

(x) and testosterone (y) concentrations (figure 5C) in large follicles. Although there was a negative correlation between estradiol-17β and testosterone in large follicles, it was not statistically significant.

Endocrinological identification of atresia

When the follicles with estradiol-17β/progesterone molar ratios of > 1.00 were considered non-atretic, and the rest were considered to be at different stages of atresia, 197/208 (95%) follicles were found to be atretic. The proportion of atretic follicles, which was 96% (56/58), 94% (122/130) and 95% (19/20) in small, medium and large follicles, respectively, did not vary signficantly among the three size categories.

DISCUSSION

Follicular fluid concentrations of estradiol-17β, progesterone and testosterone exhibited very large variations between individual follicles of the same size category. These results emphasize the large variations which exist in the hormonal microenvironment of individual follicles, and confirm and extend the results of earlier studies in sheep (England et al., 1981), cattle (Kruip and Dieleman, 1986; Wise, 1987) and a limited number of buffalo ovarian follicles (Kulkarni et al., 1994).

Large follicles had significantly higher estradiol-17β concentrations than medium sized follicles which, in turn had signifgicantly higher estradiol-17β concentrations compared to small follicles. Estradiol-17β concentrations rose with the increae in follicular size, as indicated by a significant positive relationship between estradiol-17β concentrations and follicular diameter. These results are in agreement with earlier reports in cattle (Wise, 1987; Einspanier et al., 1993) and buffalo (Kulkarni et al., 1994; Palta et al., 1996). The follicular granulosa cells contain the androgen aromatizing system which is built up under the influence of FSH and has estradiol-17β as the major product (Moor, 1977). As the small follicles have receptors for LH mainly in the theca-interna, with very few LH receptors in the membrana granulosa (England et al., 1981), these follicles can produce androgens but cannot aromatize the androgens into estradiol-17β on a large scale. The growth and development of follicles is associated with an increase in the aromatase activity, resulting in enhanced estradiol-17β production (Tsonis et al., 1984). There is also an exponential increase in the number of granulosa cells and a concomitant increase in the follicular fluid volume in developing follicles. The follicular fluid volume has been reported to increase at a faster rate than granulosa cell number in later stages of

growth in sheep follicles (Tsonis et al., 1984). A progressive increase in follicular fluid mean estradiol-17β concentrations from small through medium to large follicles, as observed in the present study suggests an increase in estradiol-17β production during growth and development of follicles which could be due to a cumulative effect of increasing aromatase activity per cell and increasing cell number (Tsonis et al., 1984) with the increase in follicular size.

Mean progesterone and testosterone concentrations were not found to be different among the three size categories of follicles in the prsent study. This is in agreement with some earlier reports in cattle (Einspanier et al., 1993) and buffalo (Kulkarni et al., 1994) but differs from others (Nigam et al., 1994) in which small follicles were reported to have higher concentrations of testosterone compared to medium and large follicles. This discrepency could be due to the fact that in the present study the data were pooled on the basis of only follicular size, irrespective of the degree of atresia and the stage of estrous cycle, both of which also influence follicular testosterone concentrations (Kruip and Dielemen, 1986). It is now well established that both granulosa and theca cells of bovine follicles produce large amounts of progesterone which serves as a precursor for androgen and, subsequently estrogen production (McNatty et al., 1984). The results of the present study, in combination with earlier studies (Kruip and Dieleman, 1986) suggest that intrafollicular progesterone production is not significantly influenced by an increase in follicular size but may vary with degree of atresia and stage of estrous cycle.

The interpretation of the results of the present study in terms of the interrelationships between the three steroid hormones is limited by the fact that no information was available on the stage of reproduction of the ovaries, as these were obtained from abattoir. Lack of relationships between the three hormones in small and medium follicles could be due to grouping of follicles on the basis of only follicular diameter, without taking into consideration the degree of atresia and the stage of estrous cycle. A negative relationship between estradiol-17β and progesterone and a positive relationship between progesterone and testosterone concentrations in large follicles may reflect precursor-product relationship and suggests utilization of follicular pool of progesterone for testosterone production which, in turn can be utilized by an active aromatase system for estradiol-17β production.

Hormonal concentrations in the follicular fluid can be used to provide information on follicular health as estradiol-17β/progesterone and estradiol-17β/testosterone + androstenedione ratios have been reported to be negatively correlated with atresia in cattle (Ireland and Roche, 1982). Mukhopadhyay et al. (1991) found 90% of histologically characterized follicles to have an estradiol-17β : progesterone ratio of < 1. In the present study, when the follicles were characterized on the basis of the molar ratios of estradiol-17β/progesterone in individual follicles, 95% follicles were found to be atretic in abattoir ovaries at random stages of reproduction. Ocampo et al. (1994) have also reported a similar figure of 82% on the basis of histological examination of follicular atresia in swamp buffaloes.

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