Changes in Steroid Receptor Number of and Bioactivity of Gonadotropin in the Follicular Fluid of Porcine Ovarian Atretic Follicles I. Bioavailable Testosterone

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The present study was disigned to determine the concentration of bioavailable steroid hormones in the atretic follicular fluid (FF). The concentrations of progesterone (P), testosterone (T), estradiol (E), androstenedione (A), and 5-α dihydrotestosterone (DHT) were determined by the established methods of luminescent immunoassay (LIA) or radioimmunoassay (RIA). Concentrations of T, A and DHT in human FF from small ($<$ 6 mm), medium (8-15 mm), and large ($>$ 15 mm) atretic follicles were significantly higher than those of normal ones ($p <$ 0.01). However, the levels of T, A and DHT in small atretic follicle were significantly lower than those found in normal one. The concentrations of P in atretic FF from porcine small ($<$ 3 mm), medium (4-6 mm), and large ($>$ 7 mm) follicles were not different from that of normal ones. However, the concentration of E in atretic follicles of each group was significantly lower than that of normal group ($p <$ 0.001 in each group). On the other hand, the percentages of bioavailable T (BT) in human FF were significantly ($p <$ 0.001) higher than those in normal groups. The BT in normal or atretic FF was more than 90 % of total T.

The present result demonstrates that the bioavailable androgen, but not E levels in atretic follicles is higher than that of normal one, and that the atretic mechanism might be dependent on the ovarian follicle size in the developmental stage and on the animal model system. Moreover, the present study suggests that the steroids found in the FF are the bioavailable forms and the concentration of BT in FF could be used as one of the valuable criteria classifying the ovarian atretic follicle.

KEY WORDS; Atresia, Bioavailable testosterone

Steroid hormone is the microenvironmental factor for the cumulus enclosed oocyte and the local regulator for autocrine control of the follicular growth or maturation in mammalian ovary. It is well known that antral follicular development is dependent upon intrafollicular estrogen, and that the increased aromatization of androgens by granulosa cells (GC) is the hallmark of an ovulatory follicle

(McNatty et al., 1979; Richards et al., 1986, 1987; Richards and Hedin, 1988). Recent studies indicate that the reduced aromatase activity in GC to induce lower estradiol (E) level in follicular fluid is an early event in the atretic degeneration of antral follicles (Moor et al., 1978; Carson et al., 1981; Tsonis et al., 1984; Maxon et al., 1985). Thus, the higher concentration of androgen in follicular fluid is associated with follicular atresia, while a relatively higher level of estrogen is related to a large dominant follicle maturation (McNatty et al., 1979; Bomsel-Helmreich et al., 1979; Tsonis et al.,

1984; Lee and Yoon, 1985; Lobo et al., 1985; Lee et al., 1986). Androstenedione (A) is the major steroid synthesized by theca interna tissues and converted to androgen such as testosterone (T) and 5 α -dihydrotestosterone (DHT) in the bovine ovary (McNatty et al., 1985). However, the concentration of A is not different between in the normal follicular fluid and in the atretic one as determined by the histologically classified medium-sized bovine follicles (Henderson et al., 1984, Kruip and Dieleman, 1985; Spicer et al., 1987), while A, T and DHT in human atretic follicles are increased (Lee et al., 1986). Our previous works clearly demonstrated that progesterone (P) and T in follicular fluid of porcine atretic follicles were increased (Yoon et al., 1989). On the other hand, in the rodents, a lower E or T level in atretic follicle seems to be due to the reduced production of aromatizable androgen (Tsafriri and Braw, 1984). Therefore, it can be thought that these kinds of contradictory results might be due to the differences of animal species as a model system or the collection time of samples or the size and status of maturity of follicles. Yoon et al. (1988) had reported that DHT and T in serum are largely bound to either sex- hormone- binding globulin (SHBG), and to albumin. Only 1-3 % of DHT or T is in a non-protein bound (i.e. free) state and represents biologically active. This report also suggested that the bioavailable T (bio T, free T plus albumin bound T, rather than total T appears to be correlated with androgen activity of the reproductive states.

We can assume, therefore, that only the bioavailable fractions of these steroids in follicles might act through their receptors. The determination of bioavailable androgens in follicular fluid may provide the direct measure of physiologically active androgen levels and also show the correlations with androgenecity in follicular atresia. Measurement of total T or DHT in follicular fluid may be, therefore, misleading to study the role of steroids in the follicular maturation and atresia.

Thus, in the present study, we measured the bioavailable DHT and T by a chemiluminescence immunoassays (LIA) for investigating the change in bioavailable steroid hormone concentrations in follicular fluids during the follicular maturation and atresia. Moreover, the present study was designed to know whether the bioavailable androgen could be an indicator for the follicular atresia.

Materials and Methods

Porcine ovaries were obtained from the slaughter house (Woosung Nonghyub) located at Majang-dong, Seoul. The ovaries were excised within 20 min after sacrifice and transported to the laboratory in a jar filled with sterilized and ice chilled 0.154 mol /1 sodium chloride solution containing 100 IU/ml penicillin and 100 μg/ml streptomycin or directily in ice cold container within 1 hour.

Human follicles (n = 185) from unstimulated ovaries were obtained from 86 patients at the random phase of menstrual cycle immediately after surgery from three local hospitals. The indications of surgery were as follows; ten with pelvic inflammatory diseases (mean age 41.0 ± 7.1), two with adrenal tumor (42.5 ± 0.7), twenty-three with uterine myoma (33.7 ± 8.7), and the others with unknown diseases (38.0 ± 7.5). All (n = 198) follicular fluid samples were obtained in 63 cycles from 41 women treated for IVF and ET because of the tubal blockage from Dr. DeGeyter in Muenster, FRG. The mean age of patients was 35.3 ± 5.3 years (range from 27 to 46). Ovarian hyperstimulation was performed by administrating with either hMG (Humegon, Organon, Oberschleissheim, FRG or Pergonal, Serono, Freiburg, FRG) or huFSH (Fartinorm, Serono, Freiburg FRG) as described previously (DeGeyter et al., 1988). The initial dose was maximally 5 ampoules per day and the dose was decreased depending on the serum estradiol (E) levels. The ovarian stimulation was monitored by a daily measurement of E, P, and LH in serum. Ovulation was induced by an intramuscular injection of 10,000 IU hCG (Pregnyl, Organon). The follicular fluids were aspirated by a laparoscopy at 34 to 36 hours following hCG treatment.

The porcine ovaries were grouped into follicular (NCL, corpus luteum absent) and luteal phase (CL, functional corpus luteum present) by the morphological criteria of corpus luteum, corpus albicans and the follicle size or number of large follicle as described in detail previously (Lee and Yoon, 1985). The ovarian follicles were dissected out from

the grouped ovaries and then classified into small (diameter, less than 3 mm, S), medium (3-5 mm, M) or large (6-12 mm, L) follicles after measured by a calipher. The follicles with a pale vascularization of theca layer vessels and with a large portion of black or opaque dots or with follicular fluids containing floating cell debris under the stereomicroscope (Wild M5A) were grouped into atretic follicles. To confirm and reclassify the follicular status, the follicles were slit on the wall to release the intrafollicular contents. The harvested GCs were washed twice with TC 199 culture medium, and then its viability was determined by a method of trypan blue (final dilution, 0.1 %) dye exclusion using a hematocytometer. The nuclear phase of the collected oocyte was examined by whole mount techniques described previously (Lee and Yoon, 1985).

The indirect RIAs for DHT or T in follicular fluid were performed as described previously (Yoon et al., 1981; 1987). The free- or bioavailable androgens were measured by the LIAs (Yoon et al., 1988). The between assay variations (BV) for T- and DHT-LIA were 8.7 % at 42.4 ± 3.7 pmol/1 and 7.8 % at 10.3 ± 0.8 pmol/1 respectively. The within assay variations (WV) were 6.1 % at 45.6 ± 2.8 pmol/1 for T-LIA and 5.5 % at 9.8 ± 0.4 pmol/1 level for DHT-LIA.

Human follicular FSH was measured with a commercial kit (Delfia hFSH, LKB, Turku, Finland). Follicular LH and hCG in human follicular fluids were determined with Maiaclons of Serono (Serono, Freiburg, FRG). Estradiol-17 beta and progesterone, androstenedione were measured by the RIAs as shown in the previous studies (Yoon et al., 1981; 1988).

Statistical analysis of the data obtained from normal subjects was done by student's t-test. The data which were not normally distributed in the samples were performed using a Mann-Whitney "U" test. The correlation between two different variables was calculated by a linear regression analysis. All results are expressed as the mean ± SD, unless otherwise stated.

Results

After isolation of the individual follicle (n = 60), the diameter of follicles (range 1~7.8 mm) was

measured and then the follicle was punctured in test tube containing 10,000 cpm tracer in 0.154 M saline. The radioactivity of 500 μl from test tube was counted. To compare with the absolute volume, the same amount of tracer (10 μl/ml) was pipetted into a tube containing the known volume of physiological saline and then the radioactivity of 500 μl was counted. The relationship between follicular diameter (D) and volume (V) of follicular fluid in porcine follicles was formulated as the following formula: $V = 0.32 \times D^3$. There is no correlationship between the diameter of follicle and the size of oocyte. To measure the density of porcine follicular fluid, the weights of 5 to 100 μl of distilled water and the same amount of pooled follicular fluid were measured with Sartorius balance (Model 2007 MP). The density of porcine follicular fluid was 1.02 ±0.04 (CV = 3.99 %).

The measured steroid concentrations in human follicular fluid are summarized in Fig. 1. The concentrations of A, T, and DHT in the hFF of large follicles (> 15 mm) from the unstimulated women were significantly higher than those in the normal ones. In small follicles (< 6 mm), the levels of P and A in hFF of atretic follicles were much lower than those in normal ones. The concentrations of P and E in hFF from all sized and the unstimulated follicles were also siginificantly lower than those from normal ones.

In the hyperstimulated follicles with hMG or huFSH, there is no difference in steroid hormones between of follicles from hMG stimulated cycles and from huFSH treated cycles (Table 1). However, the concentration of E from the follicles of hMG treated cycle were significantly higher than that of huFSH stimulated cycle. When compared with the absolute amount of steroid hormones, P (13.94 ~ 14.53 μ mol/1) in the hyperstimulated follicular fluid was 5-7 times higher than that in the large follicles of unstimulated cycle. The levels of T (6.4 ~ 7.2 nmol/l) and DHT (1.83 ~ 2.08 nmol/l) in the hyperstimulated follicular fluid were approximately 70 times lower than those in the unstimulated ones. And the level of E in hyperstimulated follicles was 5-10 times lower than those in the unstimulated ones.

Steroid hormones of the preovulatory follicles especially from unstimulated cycle and fertile

Table 1. Steroid hormone concentrations in human follicular fluid from the hyperstimulated ovarian follicle in IVF programme
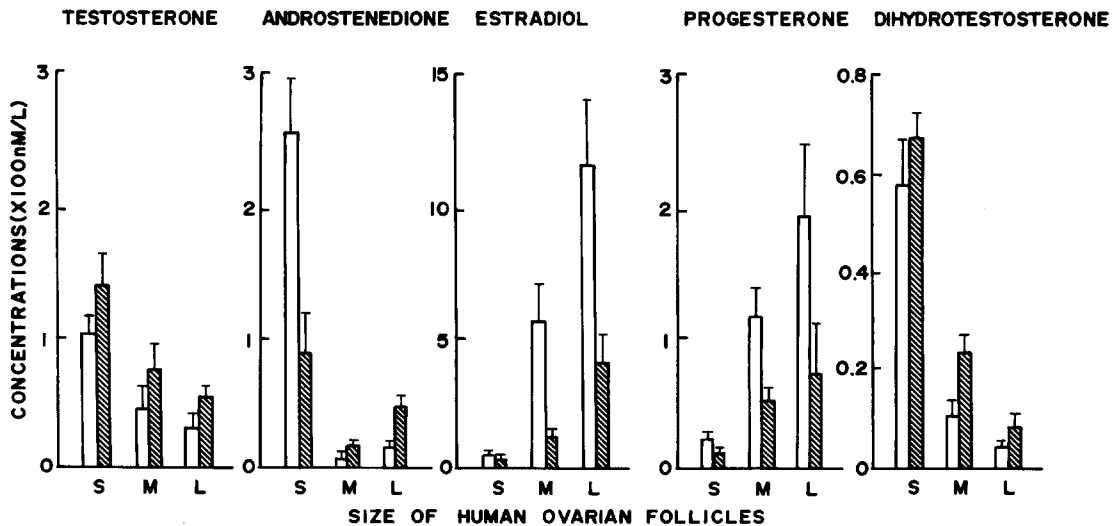
steroid hormones	Stimulations	
	hMG (n=63)	huFSH (n=74)
Progesterone (μmol/1)	13.94 ± 1.41 (3.6 − 23.3)	14.53 ± 0.79 (3.2 − 24.5)
Testosterone (μmol/1)	6.38 ± 0.65 (2.2 − 10.3)	7.21 ± 0.61 (2.1 − 11.8)
Estradiol (μmol/1)	1.69 ± 0.16 (0.7 − 2.60)	*1.28 ± 0.22 (0.5 − 2.0)
Dihydrotestosterone (μmol/1)	1.83 ± 0.32 (1.03 − 2.61)	2.08 ± 0.19 (1.29 − 2.86)

*indicates a significant difference of $p < 0.05$.

The follicles were grouped according to medication used for ovarian hyperstimulation. The figures represent the mean values ± SD with the ranges in the parentheses.

women with the regular menstrual cycle were determined. As shown in Fig. 2, the concentrations of E and P in atretic follicles (AI), whose oocytes in TC 199 medium were degenerated dicrate oocytes during 6 hour incubation under 37℃, 5 % CO_2 condition, were significantly lower than those of the normal GVBD oocytes. Concentration of T of AI was slightly higher than that of the normal one, but T was significantly higher ($p < 0.001$) in the follicles (AII) whose oocytes became necrotic, degenerating GVBD, or fragmented during incubation of 4~6 hours. The P and Econcentrations in AII follicles were significantly lower than those in the normal follicles ($p < 0.001$).

LH concentrations (mIU/ml) of the follicular fluids in the human follicles treated with hMG cycle (hMGFF) and those treated with huFSH cycle (huFSHFF) group were 3.3 ± 0.4 and 2.9 ± 0.4, respectively. FSH contents (mIU/ml) in hFF were less than 3 and FSH level in hMGFF was not different from that in huFSHFF. The concentrations of hCG (ranging 76 ~ 135 mIU/ml) in both groups

Fig. 1. Concentraitons of steroid hormones in human follicular fluid of normal and atretic follicles from the hyperstimulated ovary in IVF and ET programme.

The human ovarian follicles from hyperstimulated cycles were grouped into small (S, < 6 mm), medium (M. 8−15 mm) and large (L, > 15 mm) follicles. The concentrations of all steroid hormones were determined in the same follicles by LIA except A. The numbers of the normal (N) or atretic (A) follicles in each group were as follows: N = 21 and A = 15 in small follicles; N = 18 and A = 13 in medium group; N = 54 and A = 35 in large ones. The differences between normal follicles (white bars), and atretic ones (shaded bars) were demonstrated as the following marks: * = P <0.01; ** = P < 0.001.

were not different.

The steroid hormones in porcine follicles were determined (Fig 3). The P concentrations in small- and medium-sized atretic follicles were not different from those of normal ones, but in the large follicles, the level of P in atretic follicles was significantly lower than that in normal follicles. The concentrations of T in atretic follicles were slightly higher than those in normal ones, but not statistically significant. However, the level of E in atretic follicles was much lower than that in any sized normal follicles.

The concentration of total protein in the porcine follicular fluid was clearly (p < 0.01) increased during the early follicular growth phase and then slightly decreased in large follicular stage. The protein concentration in atretic follicles of medium size (pFFMA) in pig ovary was lower than those of normal one. The absolute concentration of total protein in the pFF was 74 % in serum and 83 % in the human follicular fluid. The absolute albumin concentrations in follicular fluid of human and of porcine follicles were 30.1 ± 8.2 and 25.8 ± 0.9 mg/ml, respectively and those in FF were 78.3 %

of human serum and 63.1 % of porcine serum, respectively.

The concentrations of hFF T which has been bound to SHBG and of the bioavailable T are summarized in Table 2. The percentages of SHBG-T were 12.8 % in normal small follicles (NS) and 11.5 % in atretic ones (AS). The bioavailable T percentage was more than 85 % in both follicles. On the other hand, the bioavailable percentages of normal large follicles and atretic ones were more than 93 % and 95 %, respectively. The statistical significance of SHBG-T and bioavailable T (BT) to the total T could not be calculated because the sample sizes were small and the variations were large. However, we found that the percentage of BT in follicular fluid was clearly higher when compared with the value (28.9 ± 4.1 %) found in serum. The BTs in atretic follicles were much higher than those in normal ones in all-sized follicles.

Fig. 3. Concentrations of steroid hormones in porcine follicular fluids of normal and atretic follicles.

The porcine follicles were isolated and grouped as shown in the text: S (small, < 3 mm; n = 34 for normal follicles (N) and n = 45 for atretic ones (A)); M (medium, 3-5 mm; n =28 for N and n= 35 for A) and L (large, > 7 mm; n = 42 for N and n = 37 for A). The atretic follicles (shaded bars) were classified by the criteria of pale vascularization of thecal layer, opaque or dark portion of the isolated follicles under stereomicroscope, oil drops and cell debris in the follicular fluids and also higher pyknotic index (> 40 %) and also with degenerating, necrotic or fragmented oocyte.
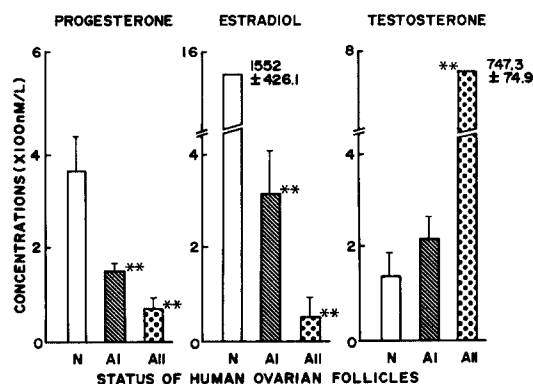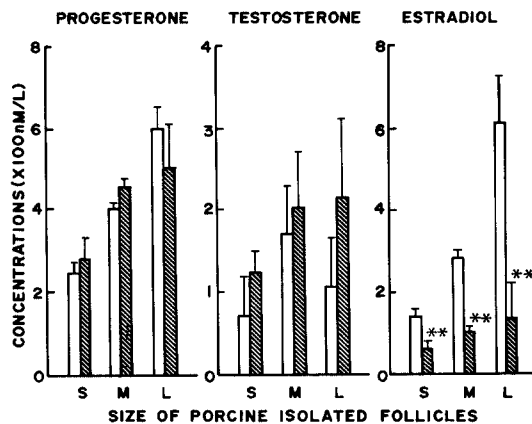

Fig. 2. Concentrations of steroid hormones in human follicular fluid of normal and atretic preovulatory follicles from unstimulated and normal menstrual cycles.

The vertical bars represent the standard deviation of the arithmatic means. AI (shaded bars) represents the initiating atretic follicles with degenerating dictyate oocytes and AII (dotted bars) include the marked advanced atretic follicles with degenerating and germinal-vesicle- breakdowned (GVBD) oocytes and also necrotic or fragmented ova.

Table 2. Bioavailable and SHBG-bound testosterone concentration in human follicular fluid

Distribution	Follicular fluids		
	small	medium	large
Total testosterone			
normal	299.1 ± 30.9	136.7 ± 37.7	91.7 ± 30.5
atretic	405.4 ± 63.9	219.4 ± 50.8	161.3 ± 16.5
SHBG-bound testosterone			
normal	38.5 ± 16.7	15.3 ± 8.5	5.14 ± 2.7
atretic	46.6 ± 21.3	28.9 ± 31.3	9.0 ± 18.5
Bioavailabel testosterone			
normal	250.5 ± 68.7	109.5 ± 23.4	85.4 ± 15.5
atretic	360.5 ± 130.2	199.3 ± 87.2	153.5 ± 26.7

The data represent mean (nmol/1) ± SD. The atretic follicle (AI +AII) was grouped according to the criteria as shown in Fig. 2. Bioavailable T was measured after SHBG-T was precipitated by ammonium sulphating (final 50%). The numbers of the measured samples are shown in Fig. 1.

Discussion

The present study denied the notion that progesterone / androgen-dominant follicles with a low estradiol condition might be classified into atretic follicles except in large follicles of human ovary (McNatty et al., 1979; Spicer et al., 1987). Instead, the present study showed that in human atretic follicles, androgen-dominant, progesterone and estrogen depleted follicles are the atretic follicles. These results of human follicles are not agreed with those of porcine follicles, but at this moment it is not clear whether this different result comes from the species-specificity.

It has been reported that the concentration of progesterone in large follicles is increased during atresia in cattle (Ireland and Roche, 1982; Bellin and Ax, 1984; Kruip and Dieleman, 1985), but not changed in sheep (Moor et al., 1978) and in human (Bomsel-Helmreich et al., 1979; McNatty et al., 1979; McNatty, 1981). In hamster and rat follicles, the level of progesterone is increased, decreased, or remain unchanged (Tsafriri and Braw, 1984; Na et al., 1985). Our results suggest that this discrepancy may depend on the model system used, timing of sample collection and the definition of atresia. We already reported that there are several stages of the

follicular atresia from initial stages to cystic stages (Kim et al., 1987). This concept could be supported by the results of the previous work (Spicer et al., 1987).

Most of the previous studies agreed well with the notion that in the primates the level of estradiol in atretic follicles is low, and that it may be due to a lower aromatase activity (Maxon et al., 1985; Spicer et al., 1987). On the contrary, Tsafriri and Braw (1984) reported that in rodents, a lower estradiol level is due to the reduced production of aromatizable androgens. Our results appear to support the former notion because androstenedione, one of the major substrates for aromatase and testosterone in atretic follicles were higher than those of normal ones.

Some reports showed that only 2.0 and 3.6 % of collected human oocytes have GVBD oocytes (McNatty et al., 1979) and 7 % of oocytes in small follicles have GVBD ova (Gougeon and Testart, 1986). The percentage of GVBD was increased with the degree of follicular atresia, especially, during the period of ovulation. These results indicate that the human follicles with GVBD oocytes in the present study were atretic follicles. The mechanism of GVBD in human atretic follicles is not clear, but it seems that the follicular atresia could induce the removal of inhibited nuclear division of the oocytes. However, Cran et al., (1983) did not found the

spontaneous resumption of oocyte meiosis *in vivo* in sow atretic follicles, although we scarcely observed. This discrepancy and the mechanism to induce the resumption of arrested meiosis of oocytes in atretic follicles remain to be elucidated.

It has been reported that there is no differences of SHBG binding affinity during the reproductive status. However, the gender difference in SHBG emerges during the prepubertal development, but normal decreases in SHBG levels of hypogonadal human males and androgen insensitive individuals are occurred (Anderson *et al.*, 1974). These results suggest that factors besides the sex steroids may be responsible for this change (Cunningham *et al.*, 1984). Thyroid hormone increased SHBG and obesity is associated with the decreased SHBG (Siiteri *et al.*, 1982). and GH may be involved in the regulation of SHBG (De Moor *et al.*, 1972).Because the some components of follicular fluid are the same as those of serum, we can assume that SHBG in follicular fluid may be changed by these factors mentioned above, but it remains to be elucidated.

The present study demonstrated that the bioavailable testosterone is more than 80 % of total testosterone and represents the active form in the follicular fluid of mammalian follicles. In serum, 98 % of circulating testosterone is bounded to SHBG and albumin (Anderson, 1974; Manni *et al.*, 1985; Yoon et al., 1988). Because SHBG concentration in follicular fluid of mammalian follicles is limited, the BT in follicular fluid is especially free testosterone and its percentage seems to be higher than that in serum. Present study, therefore, suggests that more than 85 % would be secreted into the blood circulation, and that changes of BT might reflect the follicular steroid environment.

We measured steroid hormone receptors in granulosa cells from the porcine follicles (Yoon *et al.*, 1989). These results suggest that the androgen treatment induces the decrease in ovarian weight by the reduction of estradiol receptors in the ovary, whose follicles showed the initial stage of atresia. Based upon these results, we could assume that the higher concentrations of androgens in the mammalian atretic follicles might reduce the induction of estradiol receptors. The relationship between steroid levels and the concentrations of steroid receptors remains to be elucidated.

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돼지의 폐쇄여포내 스테로이드 수용체의 변화와 여포액내 생식소자극 호르몬의 활성도 변화
I. 활성적 Testosterone의 농도

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포유동물의 생식주기중 대부분의 여포는 폐쇄되고 극히 일부만 배란된다. 돼지의 폐쇄여포액내에는 다량의 androgen이 존재하며 이들이 폐쇄의 한 요인이라는 결과(Lee and Yoon, 1985)로 보아 폐쇄여포액내에는 다량의 활성적 testosterone이 존재할 것으로 추론하고 이를 정량하고져 하였다. 사람의 여포액내 progesterone (P), testosterone (T), estradiol (E), androstenedion (A)과 dihydrotestosterone (DHT)의 농도를 본 연구에서 정립된 섭광면역측정법 및 방사면역측정법으로 측정한 결과는 다음과 같았다. 사람의 폐쇄된 여포액내 T, A, DHT의 농도는 소(6mm 이하), 중 (8—15 mm), 대 (15 mm이상)여포 공히 정상의 여포액내 이들 스테로이드의 농도보다 현저히 증가되어 있었다($p < 0.01$). 그러나 소여포의 경우 폐쇄여포액내 A의 농도는 정상에 비하여 현저히 낮은 농도를 나타내였다($p < 0.001$). 반면 P와 E의 폐쇄여포액내 농도는 정상에 비해 현저히 낮은 농도를 나타내었다. 돼지의 소(3mm이하), 중(3—5mm), 대(7mm이상)의 폐쇄여포액내 P의 농도는 정상과 통계적으로 유의한 차이가 없었다. T의 경우 역시 각 각의 크기를 가신 폐쇄여포액에서 정상 여포액 농도보다 높게 나타났으나 통계적인 유의성은 없었다. 그러나 E의 경우는 폐쇄여포액내 농도가 정상에 비해 현저히 낮게 나타났다. 한편 여포액내 활성적 T의 점유율(%)은 혈청에 비해 현저히 높았다($p < 0.001$). 즉 bioavailable T (BT)는 정상과 폐쇄여포액내에서 각기 90 %이상을 나타내었다.

위의 결과를 종합해 볼때 동물종, 크기에 따라 폐쇄의 요인은 다른 것으로 사료되며 폐쇄여포액내 활성적 androgen의 농도는 정상에 비해 높고 또한 E의 농도는 현저히 낮은 것으로 나타났다. 또한 여포액내 대부분의 스테로이드는 활성형의 상태로 존재하며 활성적 T는 폐쇄여포의 판정에 주요기준이 될 수 있을 것으로 판단된다.