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Follicular Lactate Dehydrogenase Activity and Steroid Concentrations in the Immature Gilt Ovary

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미성숙 돼지 난포 내 Lactate Dehydrogenase 활성도 및 동일 난포액 내 스테로이드호르몬의 농도변화

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연구목적: 난포가 폐쇄되는 동안의 생화학적 변화를 규명하기 위하여 미성숙 돼지의 정상 및 폐쇄 난포 내 lactate dehydrogenase (LDH) 활성도 변화 및 동일 난포액 내 스테로이드호르몬의 농도변화를 알아보기 위하여 본 연구를 시행하였다.

재료 및 방법: 난포액 (FF), 과립세포 (GC), 협막세포 (TC) 내 LDH의 활성도를 측정하였으며, 난포액 내 progesterone (P_4), testosterone (T), estradiol (E_2)의 농도변화를 방사면역측정법으로 정량하였다.

결 과: 정상 및 폐쇄 난포에서 P_4의 농도변화를 보이지 않았다. 그러나 폐쇄 난포액 내 T의 농도 (3.85±1.50 ng/ml)는 정상 난포 (1.29±0.54 ng/ml)에 비해 현저히 높았으며 정상 난포 내 E_2의 농도 (43.29±19.51 ng/ml) 는 폐쇄 난포 (18.82±7.27 ng/ml)에 비해 현저히 높은 것으로 나타났다. 정상 난포액 내 P4의 농도는 난포의 크기에 정의 상관관계 (r=0.75)를 보였다. 정상 난포 내 T:P_4의 비율 (8.14±3.35)은 폐쇄 난포 (1.39±0.60)에 비해 현저히 높았으며, 정상 TC (433.63±102.40 μU/μg DNA) 및 FF (246.86±58.96 μU/μl) 내 LDH 활성도는 폐쇄 난포 (각각 83.7±10.5와 38.71±9.00)에 비해 현저히 높게 나타났다. 정상 난포의 GC 및 FF 내 LDH 활성도는 E_2의 농도와 부의 상관관계를 보였지만, 폐쇄 난포의 TC 내 LDH 활성도는 P_4, T, E_2의 농도에 대해 정의 상관관계를 나타내었다.

결 론: 본 실험의 결과, 미성숙 돼지 난포의 폐쇄는 TC 내 LDH 활성도 감소와 밀접한 관계를 갖는 것으로 사료된다.

Key Words: Follicle, Lactate dehydrogenase, Steroid hormones, Pig, Ovary

Ovarian follicular growth and development is an integrated process encompassing both extraovarian signals and intraovarian factors.[1] Follicular atresia is one of universal phenomena in female reproduction. More than 99% of total ovarian follicles are destined never to ovulate but to undergo atresia. Atresia occurs at all stages of follicle development; the penultimate stage of follicle growth is the major branching point for cohorts of developing follicles.[2] As follicular phases and reproductive cycles, steroid concentrations in the follicular fluid (FF) become changed.[3~5] At developing stage of follicles, high level of estrogen is maintained in FF,[6] and estrogen acts as one of follicle growth

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factors.[7~10] Atretic follicles show peculiar characteristics such as irregular synthesis of androgen and estrogen in FF.[11] Atretic follicles exhibit a decreased estrogen production and a lower estrogen-to-androgen ratio in FF, suggesting the importance of local estrogens for the maintenance of healthy follicles.[12] It is assumed that the irregular steroidogenesis[11] or unresponsiveness of follicles to gonadotropins[13] make the follicles become atretic. Nowadays, it is thought that the follicular atresia is processed via an apoptotic change in granulosa cells (GC)[14~17] and it is now accepted that GC pyknosis is one aspect of apoptotic process.[18]

The activity of LDH in FF of human atretic follicles became profoundly weaker when compared to that of healthy ones.[18] LDH converts L-lactate to pyruvate with NAD as a coenzyme.[19] It was reported that LDH activity was detected in theca interna of human ovarian follicle.[20] Caucig *et al*.[21] and Breitenecker *et al*.[20] reported that LDH activity was weak in nonovulatory tertiary follicles, but strong in preovulatory follicles in human ovary. It was reported that LDH might have relevance to follicular steroidogenesis[22] and follicular atresia,[23] but data concerning LDH in the steroidogenic activity of prepubertal porcine ovarian follicles according to the follicle status are few.

The objectives of this study were to measure the follicular progesterone (P_4), testosterone (T), and estradiol-17β (E_2) levels, to estimate LDH activities in the same individual follicular components including theca cells (TC), granulosa cells (GC), and FF, and to evaluate the relationship between LDH activities and steroid concentrations of normal and atretic follicles in the prepubertal porcine ovaries.

MATERIALS AND METHODS

Porcine ovaries and sera were collected at a slaughterhouse in Seoul, Korea and transported in an ice-cold 0.9% saline solution to laboratory within 30 min after the sacrifices. In a cold room (4℃), ovaries absent of corpora lutea were collected and used thereafter. Individual follicle (3.0~8.0 mm in diameter) was isolated with fine forceps.[24] Follicle sizes were measured by a caliper and were snap frozen in an acetone-dry ice water bath. They were sliced in 8~10 μm thickness with cryostat (Histostat, AO, Model 975C) and stained with hematoxylin-eosin. Under a microscope (Olympus), follicles with linear membrana granulosa and without pyknotic GC were classified into a normal group, the remaining follicles into atretic one.[24] Follicles with indistinct characters were discarded in the present experiment. The number of follicles and the mean diameter were 128 and 5.01±0.65 mm, respectively.

After cryosection, remnants of follicles were placed in a test tube at -4℃. Pinched the follicles with one forceps, the other forceps gently pierced the follicles and hooked out the contents in the frozen state. After 1 ml of saline was added in a test tube, TC was thoroughly washed out. TC was transferred into another test tube. Homogenates of GC and FF were centrifuged at 1,500 × g for 10 min at 4℃ to separate from each other. The supernatant was collected and stored at -70℃ until used. TC and GC were independently homogenized (Teflon-coated homogenizer, Wheaton) and sonicated (Fisher, Sonic Dismembrator, Model 300) for 15 seconds 4 times with 15-second-interval at 4℃. Samples were collected after centrifugation (Heraeus Christ, Minifuge) at 1,500 × g for 30 min at 4℃ and stored at -20℃ until used.

The DNA content in the homogenates of TC or GC was separately determined using the method of Harris.[25] Salmon testis DNA (Sigma), as a standard solution, was prepared at a concentration of 200 μg/ml in 2 M NaCl (Merck) containing 50 mM sodium phosphate (pH 7.4, Sigma) and 2 mM EDTA (Sigma), and diluted serially from 100 μg/

ml to 1 μg/ml as working solutions. A 100 μl of DNA standard or sample solution was added to 2.4 ml of 200-fold diluted Hoechst 33258 (2-[(4-hydroxyphenol)-6-benzimidazolyl-6(1-methyl-4-piperazyl) benzimidazol], Calbiochem.) and followed by the incubation for 5 min at 20℃. With a spectrofluorophotometer (Spectrofluorophotometer SF-510, Shimadzu), the fluorescence was read (ex = 355 nm, em = 460 nm).

LDH activity was determined by the method of Pesce.[26] A 100 μl of the sample was incubated in 2.70 ml of 57.5 mM Harris buffer (pH 7.4) with a 100 μl of 5.58 mM NADH (Sigma) solution. At the end of incubation, 200 μl of 14.0 mM pyruvate (Sigma) was added. Absorbance (A) decrease for 6 min at 30 seconds interval with a spectrophotometer (Shimadzu Spectrophotometer, UV-150-02) at 340 nm was measured. LDH activities were calculated by the equation of Pesce,[26]

$$\text{i.e} \quad \text{mU} = \frac{\text{A}}{\text{min}} \times \frac{1{,}000}{6.22} \times \frac{3.1}{0.1} = 4{,}895 \times \frac{\text{A}}{\text{min.}}$$

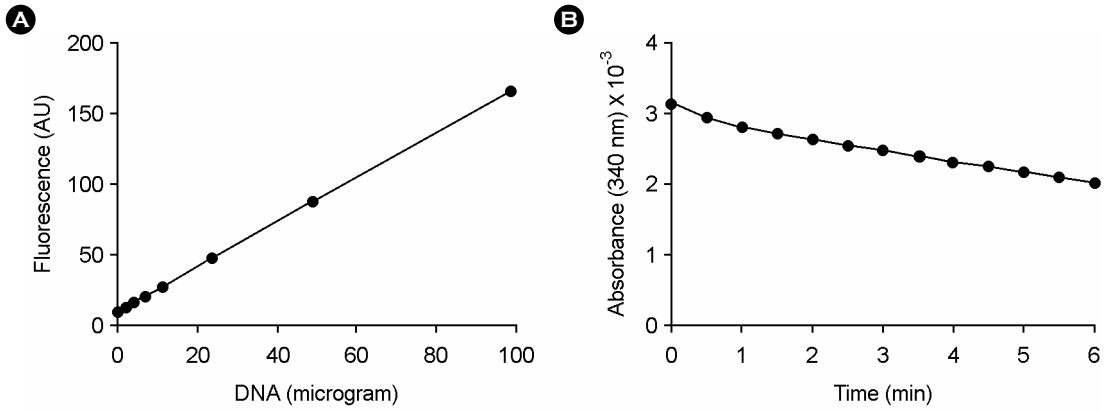

Figure 1. Typical DNA standard calibration **(A)** and LDH activity decrease curves **(B)**. DNA contents in the follicular components such as thecal layer, granulosa cell, and follicular fluid were spectrophotometrically determined using salmon testis DNA as a standard. LDH activity was determined by the method of Pesce.[26] AU, arbitrary unit.

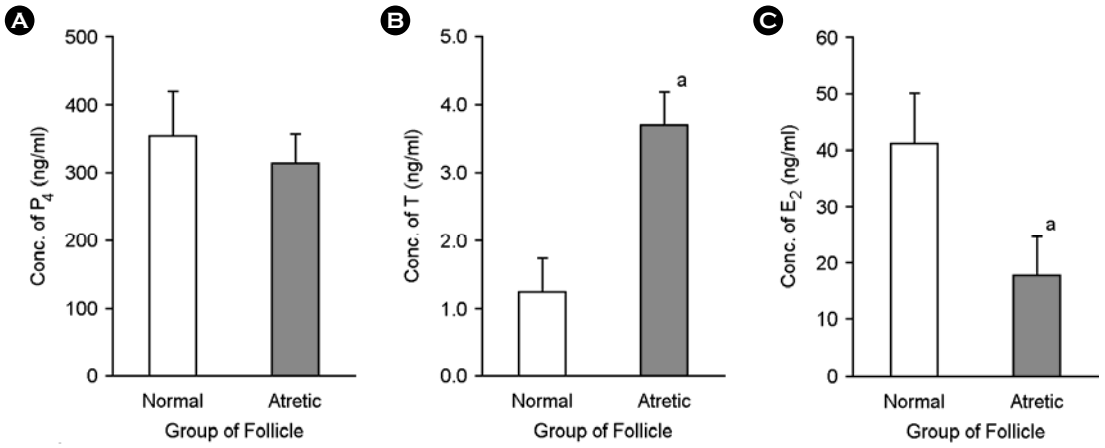

Figure 2. Concentrations of steroid hormones in the follicular fluid of prepubertal porcine ovary. **A,** progesterone (P_4); **B,** testosterone (T); and **C,** estradiol-17β (E_2). a, $p<0.05$ significantly different between normal and atretic follicles.

LDH activities of TC and GC were presented as μU/μg of DNA, and as μU/μl in case of FF and sera.

Concentrations of P_4, T, and E_2 were determined by radioimmunoassays described by Yoon *et al.*[5] Antisera of P_4, T, and E_2 were raised with P_4-2-carboxymethyl oxime (CMO)-bovine serum albumin (BSA) (titer, 1/35,000), T-3-CMO-BSA (titer, 1/84,000), and E_2-6-CMO-BSA (titer, 1/10,500), respectively. The intra- and inter-assay coefficients of P_4, T, and E_2 were 17.4% and 9.6%, 10.4% and 7.8%, and 9.5% and 18.4%, respectively. Steroid concentrations were presented as ng/ml of FF.

All data were expressed as mean ± standard error of the mean and statistically analyzed with the student's t-test. Data were also fitted to the general linear model by a least square method. The statistically significant differences were recognized at $p<0.05$ level.

RESULTS

The typical DNA standard calibration and LDH activity decrease curve were shown in Figure 1. Mean LDH activity of the immature porcine sera (μU/μl) was 800.26±52.06 (n = 12).

Concentration of steroid hormones was depicted in Figure 2. There were no differences of P_4 concentrations in FF between normal and atretic follicles. T concentration in normal FF, however, was significantly lower than that in atretic ones ($p< 0.05$). On the other hand, concentration of E_2 in atretic follicles was lower than that in normal ones ($p<0.05$). Concentration of P_4 in normal FF was positively correlated to the increment of follicle

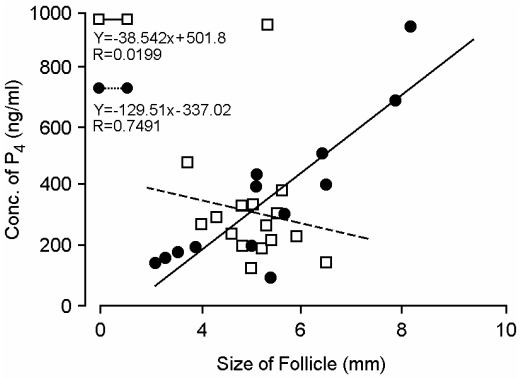

Figure 3. Correlation of progesterone (P_4) concentration to follicular size. With the increment of follicular size, P_4 concentration was increased in normal follicles (●, $p<0.05$), but in atretic ones (□), there was a no correlation between P_4 concentration and follicular size.

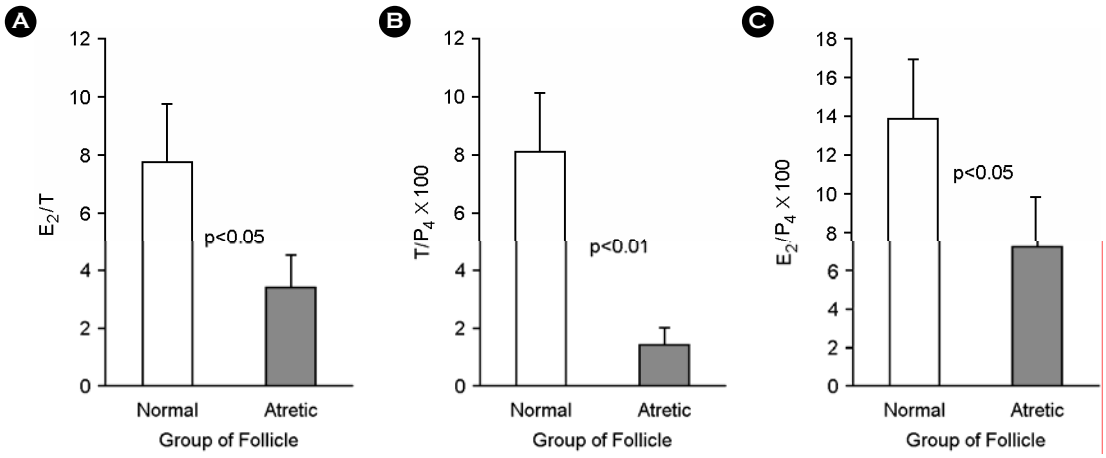

Figure 4. Concentrational ratio of steroid hormones in the follicular fluid of prepubetal porcine ovary. P_4, progesterone; T, testosterone; and E_2, estradiol-17β. **A,** concentrational ratio between E_2 to T; **B,** percentage of T to P_4; and **C,** percentage of E_2 to P_4.

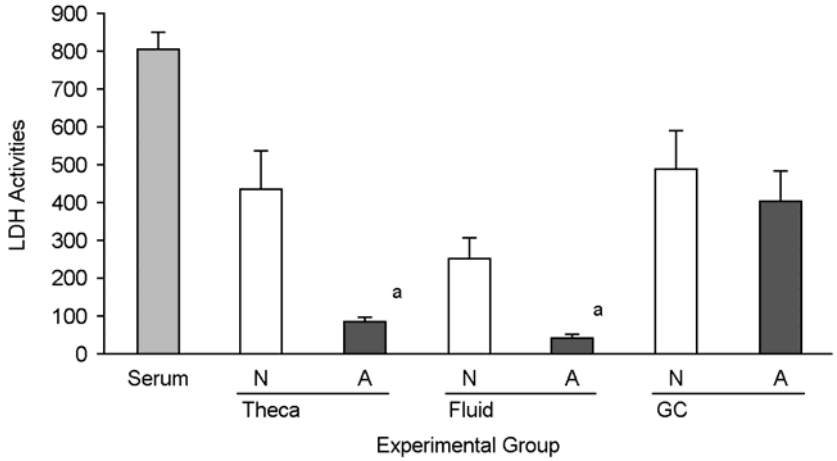

Figure 5. Lactate dehydrogenase (LDH) activities in the theca layer (TC), granulosa cells (GC), and follicular fluid (FF) of prepubertal porcine ovary and of serum. LDH activities in FF and the homogenates of TC or GC were spectrophotometrically quantified. Data are expressed as mean±SEM. a, $p<0.05$ significantly different between normal and atretic follicles. N, normal follicles; A, atretic follicles. Unit of LDH activity was μU/μg of DNA in case of GC and TC, or μU/μl of fluid or sera in case of FF and serum.

Table 1. Comparison between LDH activities in the various components of prepubertal porcine ovarian follicles and the follicular fluid steroid hormone concentrations

Follicle status	Comparison	Equation by LSM	r	p
N	GC LDH : E_2	$y = 1147.66 - 9004.27\ x$	0.90	<0.01
N	FF LDH : T	$y = 24.70 + 967.13\ x$	0.75	<0.1
N	FF LDH : E_2	$y = 403.96 - 3293.34\ x$	0.85	<0.05
A	TC LDH : P_4	$y = 62.69 + 0.82\ x$	0.87	<0.001
A	TC LDH : T	$y = 71.10 + 82.13\ x$	0.58	<0.1
A	TC LDH : E_2	$y = 75.57 + 2.72\ x$	0.80	<0.05

Abbreviations are as follows. N, normal follicle; A, atretic follicle; LDH, lactate dehydrogenase; LSM, least square method; P_4, progesterone concentration; T, testosterone concentration; E_2, estradiol-17β concentration; r, correlation coefficient; p, probability; TC, theca cells; GC, granulosa cells; FF, follicular fluid; DNA, deoxyribonucleic acid; y, LDH activity; x, steroid concentrations.

size ($p<0.05$). But in atretic FF, there were no correlations between P_4 concentrations and follicular sizes (Figure 3). Ratio of E_2 to T in normal follicles was significantly higher than that of atretic ones ($p<0.05$). Percentages of T to P_4 and E_2 to P_4 were higher in normal follicles than in atretic ones (Figure 4). LDH activities in TC, GC, FF, and sera were depicted in Figure 5. Thecal LDH activity of normal follicle was higher than that of atretic one ($p<0.05$). There were no statistical differences in LDH activity in GC between normal and atretic follicles. And it was higher in normal FF than in atretic one ($p<0.05$).

In normal follicles, LDH activity in GC had a negative correlation to E_2 concentration. But in atretic follicles, LDH in TC had positive correlations to P_4, T, and E_2 concentrations. In normal FF, LDH activity was negatively correlated to E_2 concentration (Table 1).

DISCUSSION

To know the biochemical changes on atresia, the relationship between LDH activities and steroid concentrations in the individual follicles which were histologically identified as normal or atretic in prepubertal gilt ovaries was assessed. In the present experiments, we identified the relationships between follicular LDH activity and fluidal steroid hormone concentrations. Apparently, LDH activities in TC and FF in the normal follicles were higher than those of atretic ones. This represents one of the biochemical changes of atretic follicles. Antral steroid hormones have pivotal roles in the regulation of their own synthesis and follicular development.[27,28] The concentration of E_2 in atretic follicles was significantly lower than that of in normal ones. Decrease of estrogen content in atretic follicles reflected the reduction of aromatase activity.[29] There is a possibility that high androgen concentration in the atretic FF resulted not from the elevated synthesis of androgen but from the decrease of aromatase activity. It is thought that decrease of LDH activity in atretic TC have a relevance to GC aromatase activities.

Significantly higher E_2 concentration and lower T concentration in normal FF were notified in comparison to those in atretic one. TC had a potency of T production even in atretic follicles. Furthermore, GC and TC could produce E_2 in normal follicles and in atretic ones as well.[30] There maintained a relatively high E_2:P_4 ratio in normal follicles, whereas the ratio was low in atretic ones. A low E_2:P_4 ratio was indicative of atretic status of the follicle.[31] High ratio of T:P_4 appeared in normal follicles, not in atretic ones. It might be thought that T had the important role in the folliculogenesis as reported by Hillier.[32]

In view of the finding that TC also took part in follicular estrogen production,[33,34] increase of androgen in FF of atretic follicles might result from the decrease of LDH activity. In the present study, P_4 concentration significantly increased in normal follicles as the follicle size. At midluteal phase in pigs, mean follicular P_4 concentration was higher in atretic follicles than that in normal ones.[35] This represents that P_4 might be accumulated in atretic follicles. But, there was no statistical difference in P_4 concentrations between normal and atretic FF in the present experiment. It was thought that the prepubertal ovarian follicles were not affected by gonadotropins during their early development. The previous hypothesis that the follicles with androgenic environment became atretic[36] was supported by our present results. In the present experiment, the decrease of LDH activity in TC rather than in GC had a strong relevance to follicular atresia. Wise[23] reported that LDH activities in FF increased in the atretic follicles of bovine ovary. In normal TC, LDH showed a high activity. It was thought that LDH in TC had a strong relevance to the steroid biosynthesis. Though no morphological changes of TC were observed during early atresia,[37,38] the physiological changes including the decrease of LDH activity caused the follicles to be atretic. LDH activity in GC had a negative correlation to E_2 concentration in normal follicles. But in case of atretic follicles, LDH activity in TC was positively related to E_2 concentration. Also, LDH activity in normal FF had a negative correlation to E_2 concentration. Soliman and Walker[22] reported that, in an immature rat treated with pregnant mare's serum gonadotropin, there were increments of LDH activity and E_2 concentration in the serum. It was suggested that LDH activity had a relationship to E_2 secretion. A negative correlation between LDH activities in GC or TC and E_2 concentration in the present study indicated an increase of steroid metabolism rather than an inhibition of E_2 biosynthesis by LDH. The positive relationship between LDH activities and the steroid concentration means that

a metabolic disturbance of steroid hormones in FF might be one cause of atresia or result from the physiological changes of follicular milieu.

In summary, decreases of LDH activity in TC, not GC, are closely related to the follicular atresia in the immature gilt ovary.

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