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CONCENTRATIONS OF ESTRADIOL-17β (E_2), PRO-αC, IR-INHIBIN (INH), INHIBIN A AND INHIBIN B IN FOLLICULAR FLUID OF THE GTCT AFFECTED OVARY

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ABSTRACT

To clarify the characteristic of follicular fluid in mares with granulose theca cell tumor (GTCT) 5 mares from five GTCT affected mares were obtained at the time of removal of affected ovary from different parts in Japan. The 9 ovaries from the 6 normal mares were obtained from the abattoir. The diameter of each follicle in the ovaries was measured and the follicles were classified according to the diameter. The concentration of estradiol -17β (E_2), ir- INH, proα-C and INH-A were significantly lower as compared with that of normal ovarian follicular fluid. But the average concentration of INH-B in affected animal was not significantly lower as compared with that of healthy ovarian follicular fluids. According to this study it seems that the large follicles in GTCT affected ovaries were not mature like a healthy follicle. As a result estradiol -17β (E_2), ir- INH, proα-C and INH-A were significantly lower as compared with that of normal mares.

Key words: Follicular level of FHS, LH, estradiol -17β (E_2), ir- INH, proα-C, INH-A, INH-B, Mare

INTRODUCTION

Granulosa theca cell tumors (GTCT) are the most common ovarian neoplasm observed in mares (Kaneko *et al.*, 1993). Typically, mares are diagnosed as GTCT after examination for abnormal sexual behavior, especially prolonged estrous, anestrous and stallion like behavior, by rectal palpation, ultrasonography, hormonal levels and pathological studies. Rectal palpation usually reveals one enlarged ovary and one small inactive ovary. The affected, enlarged ovary is generally composed of multiple fluid filled cysts lined by granulosa cells by ultrasonographic examination. Neoplastic granulosa cells are pathologically present as irregular shaped masses (Piquette *et al.*, 1990; Sheena and Hrapchak, 1987). However, it is unknown which kind of hormones, especially inhibin, are produced and secreted in the multiple fluid filled cysts. On the other hand, it is postulated that the contralateral ovary is inactive due to increased inhibin secretion from the GTCT, which suppress FSH secretion (Moore *et al.*, 1994). Inhibin is a glycoprotein hormone of gonadal origin that selectively suppresses FSH secretion from the pituitary gland. Inhibin A (or B) is a dimeric glycoprotein hormone composed of α and βA (or βB) subunits joined by disulphide bonds. Subunits are produced as precursor proteins that may be further processed at various clevage sites, giving rise to multiple molecular weight forms. Free α^- and βA^- subunits, and αβA, are present in equine follicular fluid (Hoque *et al.*, 2003). Free inhibin α^-subunits are also present in the circulation of various species, including horses (Nagamine *et al.*, 1998). In cattle, free α^- subunits are not capable of decreasing of FSH secretion (Hamada *et al.*, 1989). From the results of Goh *et al.* (2000) it was suggested that the concentrations of estradiol-17β (E_2) and immunoreactive-inhibin levels were lower in Case Nos. 1, 2, 4, 5 and 9 as compared with that of normal mares. The aim of the present study is to investigate the levels of estradiol -17β (E_2), pro-αC, inhibin A and inhibin B in the follicular fluid of the GTCT affected ovary, because it has not been known about the pattern of hormonal secretion in follicular fluid of the GTCT affected ovary. And, it will be discussed the relationship of hormonal levels, especially E_2 and inhibin, between in peripheral blood and in follicular fluid of the GTCT mares.

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MATERIALS AND METHODS

Five ovaries from 5 GTCT affected mares (Case Nos. 1, 2, 4, 5 and 9) were obtained at the time of removal of affected ovary from different parts in Japan. The 9 ovaries from the 6 normal mares were obtained from the abattoir. The diameter of each follicle in the ovaries was measured and the follicles were classified according to the diameter: small follicles ≥2.0 cm (n = 19); medium follicles 2.1-2.9 cm (n=15) and large follicles ≥3.0 cm (n = 11) from 5 GTCT affected mares. And also large follicles (n = 3), medium follicles (n=3) and small follicles (n=3) were collected from 6 normal mares (Table 1).

Table 1. Number of follicles used in hormonal assay

Case No.	Estradiol-17β (E_2)	ir-inhibin	Pro-αC	Inhibin A	Inhibin B
1	6 (0☆, 3•, 3♦)	6 (0, 3, 3)	------	4 (0, 2, 2)	4 (0, 2, 2)
2	9 (3, 3, 3)	9 (3, 3, 3)	-------	6 (2, 2, 2)	6 (2, 2, 2)
4	8 (2, 3, 3)	8 (2, 3, 3)	3 (1, 1, 1)	8 (2, 3, 3)	8 (2, 3, 3)
5	3 (0, 0, 3)	3 (0, 0, 3)	-------	3 (0, 0, 3)	3 (0, 0, 3)
9	10 (3, 3, 4)	10 (3, 3, 4)	3 (1, 1, 1)	9 (3, 3, 3)	9 (3, 3, 3)
Total	36	36	6	30	30
Normal	9 (3, 3, 3)	9 (3, 3, 3)	9 (3, 3, 3)	8 (3, 3, 2)	8 (3, 3, 2)

Small follicles (< 2.0 cm), Medium follicles (2.1-2.9 cm), large follicles (> 3.0), ♦Shows small sized ovarian follicle, •shows medium sized ovarian follicle and ☆shows large sized ovarian follicle.

Follicular fluid was collected by aspiration from each follicle and the fluid samples were stored at −20°C until assay. The concentrations of immunoreactive (ir−) inhibin (INH) in the follicular fluid samples were measured by radioimmunoassay using a rabbit antiserum against bovine inhibin (TNDH-1) and ^{125}I- labeled 32 KDa bovine inhibin, as described by Hamada *et al.*, (1989). The results were expressed in terms of 32 KDa bovine inhibin as shown in Fig. 1. The concentrations of estradiol −17β (E_2) were determined by double antibody RIA systems using ^{125}I- labeled radioligands as described by Taya *et al.* (1985) as shown in Fig. 1. The intra- and inter - assay coefficients of variation were 6.3% and 15.4%.

The concentrations of pro−αC, inhibin A and inhibin B in the follicular fluid were measured by enzyme-linked immunosorbent assay inhibin pro-αC assay kit (MCA 1254 Kzz; Serotec Ltd, Oxford, UK), inhibin A assay kit provided by Dr. N.P. Groome, Oxford Brookes University, UK and ELISA amplification system (Gibco BRL Ltd., USA) and inhibin B assay kit (MCA 1312 Kzz; Oxford Bio Innovation Ltd., Oxford, UK). The slopes of the dilution curves of the follicular fluid samples were parallel with the standard curves of inhibin A, inhibin B and pro−αC in all the assays.

The data were presented as the mean ± SEM. Significance between means was determine by Student's '*t*' test.

RESULTS AND DISCUSSION

The average concentrations of E_2, ir-INH, pro-αC, inhibin A and inhibin B in follicular fluid are shown in Table 2.

Table 2. Concentration of Estradiol-17β (E_2), ir-inhibin, Pro-αC, Inhibin A, Inhibin B in GTCT affected ovarian follicular fluid as compared with that of normal mares

Case No.	Estradiol-17β (E_2) Mean (min~max)	ir-inhibin Mean (min~max)	Pro-αC Mean (min~max)	Inhibin A Mean (min~max)	Inhibin B Mean (min~max)
1	3.5 ng / ml (0.6~10.8)	50.8 ng / ml (42.0~65.3)	----------	26.7 ng / ml (18.8~37.7)	0.057 ng / ml (0.019~0.12)
2	1.4 ng / ml (0.8~3.7)	59.6 ng / ml (43.4~67.9)	----------	48.1 ng / ml (42.3~54.1)	0.84 ng / ml (0.45~1.1)
4	12.5 ng / ml (5.9~29.8)	55.9 ng / ml (37.9~76.8)	37.5 ng / ml (21.7~67.5)	50.7 ng / ml (28.8~82.3)	1.3 ng / ml (0.16~5.7)
5	16.3 ng / ml (11.0~25.6)	58.5 ng / ml (50.0~67.9)	----------	69.1 ng / ml (66.1~70.6)	0.88 ng / ml (0.70~1.1)
9	5.9 ng / ml (1.2~12.6)	80.1 ng / ml (62.6~110.2)	99.8 ng / ml (48.7~145.6)	78.1 ng / ml (65.4~82.2)	2.4 ng / ml (0.80~4.5)
Normal	1181.2 ng / ml (8.9~4591.2)	515.3 ng / ml (176.0~797.9)	198.8 ng / ml (20.9~509.8)	1110.9 ng / ml (194.9~2475.0)	110.5 ng / ml (3.6~615.6)

The average concentrations of E_2 in follicular fluid of the GTCT affected mares were 30 ng / ml or less. The concentrations of E_2 in normal ovarian follicular fluids were 1,181 ± 492.1 ng / ml. The concentrations of E_2 in the GTCT affected mares were significantly lower ($p < 0.05$) as compared with those of normal mares (Fig. 1). The concentrations of ir-INH in follicular fluid of the GTCT affected mares were 50.8 ± 3.3 ng / ml in Case No. 1, 59.6 ± 2.7 ng / ml in Case No. 2, 55.9 ± 4.8 ng / ml in Case No. 4, 58.5 ± 5.2 ng / ml in Case No. 5 and 80.1 ± 4.2 ng / ml in Case No. 9, respectively. The average concentrations of ir-INH in normal ovarian follicular fluids were 515.3 ± 66.4 ng / ml. The concentrations of ir-INH in the GTCT mares were significantly lower ($p < 0.01$) as compared with those of normal mares (Fig. 1).

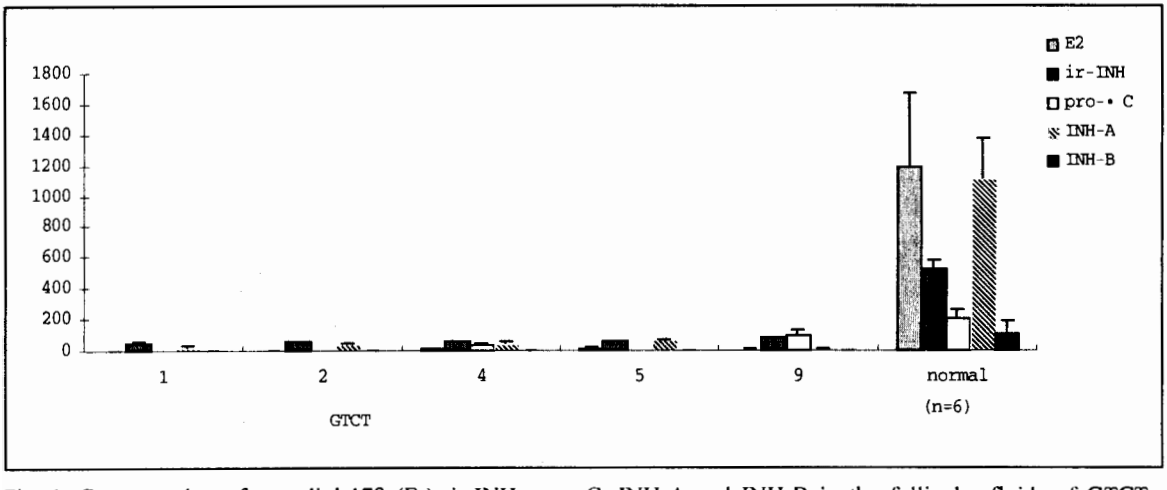

Fig. 1. Concentration of estradiol-17β (E_2), ir-INH, pro-αC, INH-A and INH-B in the follicular fluids of GTCT affected ovary

There are significant difference between GTCT affected ovary and normal ovarian follicles. The concentration of estradiol-17β (E_2), ir-INH, pro-αC and INH-A level are significantly lower but the concentration of INH-B level is lower but not significantly.

The concentrations of pro-αC in follicular fluids of the GTCT affected mares were 37.5 ± 15.0 ng / ml in Case No. 4 and 99.8 ± 28.1 ng / ml in Case No. 9, respectively. The average concentrations of pro-αC in normal ovarian follicular fluids were 198.8 ± 64.6 ng / ml. The concentrations of pro-αC in follicular fluids in the GTCT mares were significantly lower ($p < 0.05$) as compared with those of normal mares (Fig. 1). The concentrations of inhibin A in follicular fluids of the GTCT affected mares were 26.7 ± 4.1 ng / ml in Case No. 1, 48.1 ± 1.9 ng / ml in Case No. 2, 50.7 ± 7.1 ng / ml in case No. 4, 69.1 ± 1.5 ng / ml in Case No.5 and 78.1 ± 1.9 ng / ml in Case No. 9, respectively. The average concentrations of inhibin A in normal ovarian follicular fluids were 1,110.9 ± 267.4 ng / ml. The concentrations of inhibin A of follicular fluids in the GTCT affected mares were significantly lower ($p < 0.01$) as compared with those of normal mares (Fig. 1). The concentrations of inhibin B in follicular fluids of the GTCT affected mares were 0.057 ± 0.023 ng / ml in Case No. 1, 0.8 ± 0.1 ng / ml in Case No. 2, 1.3 ± 0.7 ng / ml in Case No. 4, 0.9 ± 0.1 ng / ml in Case No. 5 and 2.4 ± 0.4 ng / ml in Case No. 9, respectively. The average concentrations of inhibin B in normal ovarian follicular fluids were 110.5 ± 75.7 ng / ml. There were not significantly different in the concentrations of inhibin B between follicular fluids of the GTCT affected mares and those of normal mares (Fig. 1).

The aim of the present study was to examine the concentrations of E2, ir-INH, pro-αC, inhibin A and inhibin B in the follicular fluids of the GTCT affected mares to investigate the hormonal secretory patterns of the GTCT affected ovary. These results indicated clearly that the levels of E2, pro-αC, ir-INH, and inhibin A in the follicular fluid of the GTCT affected ovaries were significantly lower as compared with those of normal ovaries except for the concentrations of inhibin B.

From the results of Goh *et al.* (2000), it was clearly demonstrated that the concentrations of E_2 levels in the peripheral blood were significantly lower. On the other hand, it was shown that an abundant of testosterone was secreted in the present cases of mare, and was significantly higher as compared with those of normal mares. In addition, the high levels of testosterone were abruptly declined after removal of the GTCT affected ovary. It is also reported that in the case of GTCT the follicular structure are pathologically abnormal (Cordes, 1969). As the concentrations of testosterone in follicular fluid of the GTCT affected mares could not be assayed in this study, it is unknown about the source of the high concentrations of testosterone in blood. Also, until now there are no reports about the source of testosterone in mares with GTCT. However, it is reported that aromatase play an important role to convert testosterone into estrogen in healthy large follicles (Nagamine *et al.*, 1998). It seems that in the case of the GTCT the granulosa cells are unable to expressed aromatase, ultimately testosterone which are produced from the ovary cannot be converted into estrogen, and as a result it seems that the E_2 levels not only in the follicular fluid but also in the peripheral blood of the GTCT affected ovaries are low.

Nagamine *et al.* (1998) reported that the granulosa cells of follicles of all sizes and the theca cells of large follicles in normal mares are the major sources of inhibin βA and βB respectively. The concentrations of INH-A and pro-αC in the follicular fluid of large follicles also increased with follicular growth, however, INH-B concentrations in follicular fluid were not significantly different between medium and large follicles (Stabenfeldt *et al.*, 1975; Schneyer *et al.*, 1991). However, in this study the GTCT affected ovarian follicles secrete very low concentrations of pro-αC, inhibin A and inhibin B. According to these discrepancy it is assumed that in the case of GTCT, structure of the ovarian follicles with thick granulosa cell layer are not normal and the large follicles are not mature like a healthy large follicles as a result ir-INH, INH-A and Pro-αC were significantly lower and inhibin B was lower as compared with those of normal mares in this study.

Piquette *et al.* (1990) suggested that normal equine inhibin α- subunit was observe as the most prevalent 18-20 KDa, in contrast they did not observed smaller inhibin α form than 35 KDa in equine GTCT. And they also observed that 13 KDa inhibin βA subunit was observed in normal mares. On the other hand βA- subunit was approximately 13 KDa, with a minor form at 44 KDa which was observed in GTCTs. By these abnormalities, it is assumed that abnormal pro-αC, α-, βA-and βB- subunits cannot bind with each other, as a result the levels of ir-inhibin, inhibin A and inhibin B are lower in this study. It is reported in the mares with GTCT that the contralateral ovary is usually atrophied (Daels *et al.*, 1991; Evans *et al.*, 1975).

Piquette *et al.* (1990) reported that the contralateral atrophied ovary will be caused by a lack of FSH stimulation and GTCTs may produce factors that inhibit gonadotropin secretion, although they did not measure the peripheral FSH levels in their mares with GTCT. Schneyer *et al.* (1991) reported that α inhibin and or its precursors might represent autocrine or paracrine modulators of FSH action in the ovary. And Schwall *et al.* (1990) demonstrated that α- inhibin from porcine follicular fluid and human follicular fluid inhibited FSH binding to both natural tissue FSH receptors as well as recombinant rat FSH receptors. Therefore, it is possible that in the case of GTCT of mares, α- inhibin precursors (pro-αC) may inhibit FSH binding to FSH receptors in the contralateral ovary. However, in this study GTCT mares produced low levels of pro-αC. Therefore, it is unknown why contralateral ovary become atrophy as shown in Schwall *et al.* (1990). However, there are many follicles in the GTCT affected ovary. Even if the concentrations of pro-αC were lower in each follicle, it is possible that the total concentrations of pro-αC may become higher. According to these two hypotheses by the scientific proof of Schneyer *et al.* (1991), FSH cannot act on contralateral ovary, ultimately contralateral ovary become atrophic and inactive. However, it is not clear why E_2, ir-inhibin, inhibin A and inhibin B are significantly lower.

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