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CHANGES IN LEVELS AND RATIOS OF ANDROGEN, ESTRADIOL-17β AND PROGESTERONE IN PERIPHERAL PLASMA OF DAIRY COWS DURING THE FIRST 60 DAYS OF PREGNANCY[1]

JOHN YUH-LIN YU[2], JIIN-JIA LIAW, CHING-SHUENG LIN*
and MAO-CHIANG CHEN*

*Endocrinology Laboratory, Institute of Zoology, Academia Sinica,
Taipei, Taiwan 11529, Republic of China*
and
*Hsin-Chu Branch Station, Taiwan Livestock Research Institute,
Hsin-Chu, Taiwan 35099, Republic of China**

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John Yuh-Lin Yu, Jiin-Jia Liaw, Ching-Shueng Lin and Mao-Chiang Chen (1990) Changes in levels and ratios of androgen, estradiol-17β and progesterone in peripheral plasma of dairy cows during the first 60 days of pregnancy. *Bull. Inst. Zool., Academia Sinica* **29**(4): 213-222. Peripheral plasma concentrations of androgen, estradiol-17β and progesterone were measured simultaneously during the first 60 days of pregnancy in 8 Holstein-Friesian cows. Blood samples were collected at 3-day intervals during the first 30 days of pregnancy and at 5-day intervals for the next 30-day period. Plasma samples were extracted with diethyl ether and concentrations of androgen, estradiol-17β and progesterone were quantified by radioimmunoassays. Androgen remained relatively static within the range of 25 and 41 pg/ml throughout the first 60-day gestational period. A remarkable decrease in estradiol-17β concentration, from 13.1 pg/ml to 7 pg/ml, was observed during the first 3 days of pregnancy. Thereafter, the levels were maintained fairly constant between 6 and 8 pg/ml from day 3 till day 60 of pregnancy. The changes in the peripheral plasma androgen levels were quite similar to the changes in estradiol-17β levels during the first 60 days of pregnancy. Progesterone was quantitatively the highest steroid measured in the present study. It rose in a linear fashion during the first 15 days of pregnancy. Subsequently, the levels remained at a plateau of 6-7 ng/ml during days 24-60 of pregnancy.

The similarity in the secretion patterns of androgen and estradiol-17β reported in the present study, again, may implicate the role of androgen in estrogen biosynthesis. In addition to its role as an estrogen precursor, the physiological role(s) and function(s) of androgen during bovine early pregnancy, however, require further investigations.

Key words: Early pregnancy of cows, Plasma levels of gonal steroids, Progesterone, Estradiol-17β, Androgen.

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2. Reprint requests should be sent to: J.Y.L. Yu, Institute of Zoology, Academia Sinica, Nankang, Taipei, Taiwan 11529, Republic of China.

Little information is available regarding the physiological roles and functions of androgen in female reproduction. The circulating levels of testosterone and other androgens have been quantified in cows during the estrous cycle (Shemesh and Hansel, 1974; Saba *et al.*, 1975; Kanchev and Dobson, 1976; Kanchev *et al.*, 1976; Peterson *et al.*, 1978; Herriman *et al.*, 1979; Wise *et al.*, 1982; Yu *et al.*, 1988); while in pregnant cows, the androgen levels were studied primarily in late gestation as related to parturition (Heap, 1972; Hoffman *et al.*, 1976; Mostl *et al.*, 1981; Yu *et al.*, 1983; Arbeiter *et al.*, 1984). Studies concerning peripheral androgen patterns during the early stage of pregnancy were very limited (Mostl *et al.*, 1983; Arbeiter *et al.*, 1984; Gaiani *et al.*, 1984) and none of them correlated the changes in androgen levels with the corresponding secrection patterns of estrogen and progesterone.

The present study was, therefore, carried out to establish the circulating androgen, estradiol-17β and progesterone profiles of the dairy cows during the first 60 days of pregnancy. The time course relationship among these hormones may serve as a basis for understanding the gonadal steroid control in bovine reproduction during early pregnancy.

MATERIALS AND METHODS

I. Sample Collection and Storage

Holstein-Friesian multiparous cows of 4–7 years old, from Hsin-Chun station, Taiwan Provincial Livestock Research Institute, were used in this study. A total of 12 selected cows were bred during the winter months (November, 1987–February, 1988) by artificial insemination (AI) on the day of estrus (day 0). Pregnancy was confirmed by rectal palpation on days 35–40 after AI. Blood samples were taken by jugular venepuncture once every three days from day 0 till day 30, and every five days thereafter till day 60. Plasma was obtained by centrifugation and stored at −20°C until assays of steroids. Eight out of the 12 cows were identified to be pregnant; as a consequence, the circulating levels of gonadal steroids during the first 60 days of the pregnant cows were analyzed and are presented in this study.

II. Radioimmunoassays of Steroids

The blood samples were extracted with diethyl ether and allowed to freeze in a dry ice-ethanol medium. The ether was decanted into another tube and dried under ventilation at 38°C. The dried residue was dissolved in 0.01 M phosphate buffer saline (PBS) (pH 7.40) containing 0.1% gelatin and incubated at room temperature (23±2°C) for one hour. Aliquotes of the PBS-gelatin dissolved steroids were used for radioimmunoassays.

A) Androgen: Androgen was determined by a radioimmunological procedure described previously (Yu *et al.*, 1988). The antiserum was produced in rabbits by immunization with testosterone-3-CMO: BSA (Steraloids Co.). The percent cross reactions of the antiserum were: testosterone, 100%; 5α-dihydrotestosterone, 74%; androstenedione, 1.2%; 11-ketotestosterone, 0.4%; and androstenediol, 0.5%. The lower detection limit was 5 pg/assay tube. The coefficients of variation of intra- and inter-assays were 6.5% and 10.1%, respectively (N=5).

B) Estradiol-17β: The radioimmunoassay of estradiol-17β has been described previously (Yu *et al.*, 1985, 1988). The antiserum was produced in rabbits by immunization with estradiol-6-CMO:BSA (Steraloids Co.). The antibody was highly specific for estradiol-17β. The percent cross reactions relative to estradiol-17β (=100) were: estrone, 2.8%; estriol, 0.6%; estradiol-17α, 0.3%; and other steroids had negligible cross reactions with this antiserum. The lower detection limit was 1 pg/assay tube. The coefficients of variation of intra- and inter-assays were 5.5% and 12.2%, respectively (N=5).

C) Progesterone: The radioimmunoassay procedure for progesterone has been described previously (Yu and Wang, 1984; Yu *et al.*, 1988). The antibody against progesterone was produced in rabbits by immunization with progesterone-11-HS:BSA (Steraloids Co.). The lower detection limit was 5 pg/assay tube. The cross reactions of the antiserum were as follows: progesterone, 100%; 11α-hydroxpprogesterone, 62%; 11α-hydroxyprogesterone, 17%; 17-hydroxyprogesterone, 3%; corticosterone, 0.6%. The cross reactions for other steroids were less than 0.01%. The coefficients of variation of intra- and inter-assays were 6.7% and 12.3%, respectively (N=5).

Tritium-steroids [^{3}H-testosterone (1, 2, 6, 7-^{3}H(N), 112 Ci/m mole), ^{3}H-progesterone (1, 2, 6, 7, 21-^{3}H(N), Ci/m mole, and ^{3}H-estradiol-17β (2, 4, 6, 7, 16, 17-^{3}H(N)] were incubated with the aliquots of PSB-gelatin dissolved steroids from the samples, dextran-coated charcoal was employed to separate the bound-steroid from the free one. Supernatant containing the bound labeled steroids was counted in a liquid scintillation counter.

RESULTS

Changes in plasma concentrations of androgen, progesterone and estradiol-17β during the first 60 days of pregnancy are shown in Fig. 1 and Table 1. The peripheral plasma concentrations of androgen remained essentially constant, ranging between 25 and 41 pg/ml, throughout the entire 60-day gestation period, while the plasma progesterone level was the lowest at estrus and increased rapidly for the next 15 days. The progesterone level remained at a plateau, being approximately 5 ng/ml during the period between day 15 and day 21 of pregnancy, and elevated further to a second plateau of 6-7 ng/mg during day 24 and day 60 of pregnancy. As indicated in Fig. 1, the estradiol-17β level was the lowest among the three analyzed steroids. A remarkable fall in estradiol-17β concentration was observed during the first three days of pregnancy, and the level remained steadily low, between 6 and 8 pg/ml, from day 3 until day 60 of pregnancy. Such a stable secretion pattern of estradiol-17β was very similar to that of androgen during the first 60 days of pregnancy ($r=0.64$, $p<0.01$). Table 1 summarizes the circulating levels of gonadal steroids (progesterone, estradiol-17β and androgen) according to the stages of pregnancy. The steroid levels were expressed for individual sampling days during the first 9 days of pregnancy (days 0, 3, 6, and 9), since great changes occurred for both progesterone and estradiol-17β during the period. Thereafter, the steroid levels were then expressed as pooled mean values of 2-4 samplings per animal.

The ratios of circulating levels of gonadal steroids during the first 60 days of pregnancy are shown in Table 2. As indicated, four phases were discernible for the progesterone/estradiol-17β ratios: phase I (days 0-6), the ratio increased most rapidly; phase II (days 6-15), the ratio increased gradually; phase III (days 15-30), the ratio increased slightly; and phase IV (days 30-60), the ratio remained constantly high. The ratios of progesterone/androgen during the first 60 days of gestation were relatively lower than the ratios of progesterone/estradiol-17β; however, the patterns were similar to each other. The androgen/estradiol-17β ratios remained essentially constant

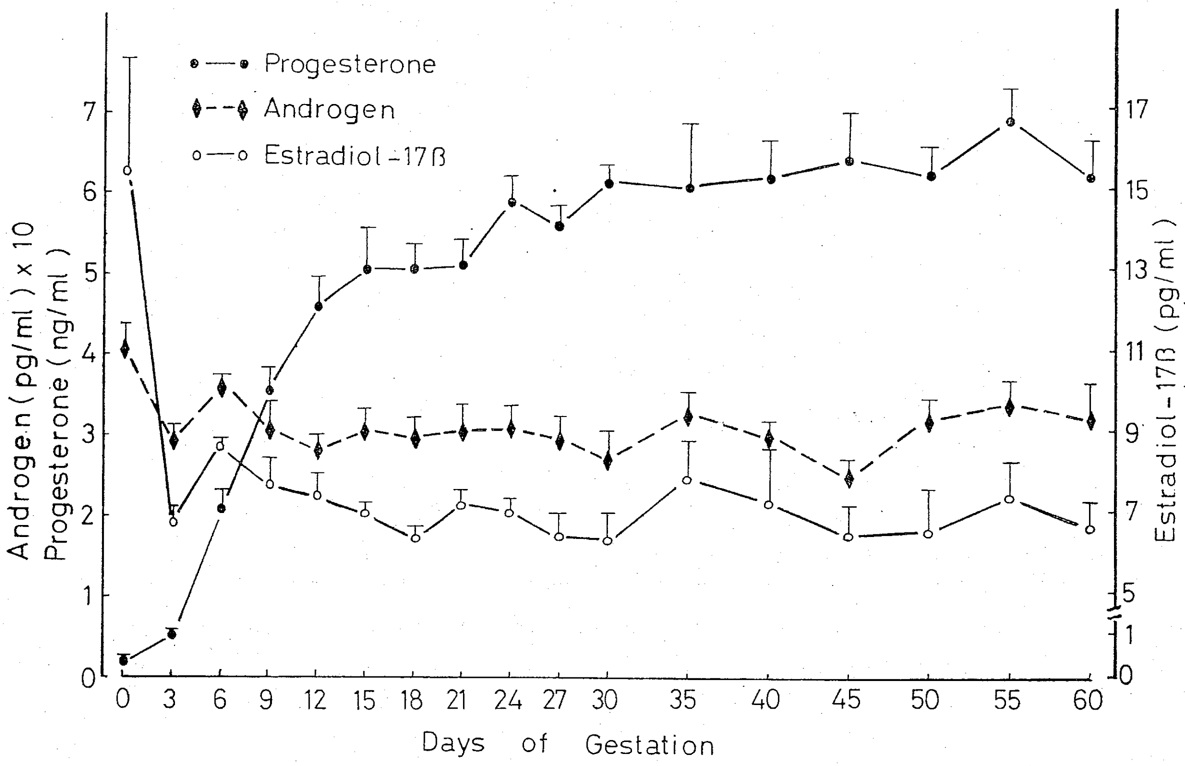

Fig. 1. Cirulating plasma levels androgen, estradiol-17β and progesterone in Holstein-Friesian cows during the first 60 days of pregnancy. The data are expressed as mean±SEM (N=8) for blood samples collected once every three days during the first 30 days of pregnancy and once every five days thereafter.

Table 1
Plasma levels of gonadal steroids in cows during early pregnancy

Stages of pregnancy (days)	No. of animals	No. of samples per animal	Progesterone (ng/ml)	Estradiol-17β (pg/ml)	Androgen (pg/ml)
0	8	1	0.23±0.07	15.50±1.62	41.00±3.30
3	8	1	0.52±0.04	6.80±0.35	29.51±1.79
6	8	1	2.09±0.22	8.04±0.31	34.90±1.90
9	8	1	3.50±0.30	7.20±0.74	31.40±2.90
12-21	8	4	4.89±0.38	6.75±0.34	29.95±3.07
24-35	8	4	5.88±0.39	6.87±0.52	30.75±3.37
40-50	8	3	6.24±0.46	6.76±0.77	28.50±2.77
55-60	8	2	6.52±0.43	7.02±0.75	32.60±3.85

The data are expressed as mean±SEM.

Table 2
Ratios of circulating levels of gonadal steroids during the first 60 days of pregnancy in eight pregnant Holstein-Friesian cows

Days of Pregnancy	Ratios of Steroids		
	Progesterone/Estradiol-17β (pg/pg)	Progesterone/Androgen (pg/pg)	Androgen/Estradiol-17β (pg/pg)
0	28± 1	10± 1	2.8±0.2
3	85± 6	18± 2	4.7±0.4
6	268±18	59± 4	4.5±0.2
9	487±13	107± 8	4.7±0.3
12	627±37	145±12	4.5±0.3
15	700±38	164±20	5.1±0.6
18	822±37	167± 8	5.0±0.2
21	811±29	162±11	5.2±0.4
24	814±39	177±14	4.8±0.3
27	864±32	195±16	4.6±0.4
30	950±67	217±21	4.5±0.3
35	923±58	193±13	5.1±0.3
40	985±39	208±11	4.9±0.3
45	996±50	215±11	4.7±0.2
50	991±61	199±16	5.0±0.3
55	1,060±89	211±14	5.2±0.4
60	986±35	193± 3	5.1±0.2

The data are expressed as mean±SEM (N=8).

during the first 60 days of pregnancy; the ratios ranged from 4.7 to 5.2 from day 3 through day 60 of pregnancy.

DISCUSSION

Pregnancy is essentially a partnership, at least from the hormonal point of view, the interactions between hormones are required for the establishment and maintenance of pregnancy. Many studies have been conducted on the secretion pattern of progesterone and/or estrogen during the first one or two months of bovine pregnancy (Donaldson *et al.*, 1970; Glencross *et al.*, 1973; Wettemann and Hafs, 1973; Eley *et al.*, 1979; Ford *et al.*, 1979). In addition, data concerning the changes in androgen levels at various stages of pregnancy of dairy cows have also been available (Heap, 1972; Hoffman *et al.*, 1976; Mostl *et al.*, 1981; Arbeiter *et al.*, 1984; Gaiani *et al.*, 1984; Yu *et al.*, 1983). However, the concurrent changes of progesterone, estrogen and androgen concentrations during the early gestational period have not been reported. This study reports, for the first time, the simultaneous determinations of the secretion profiles of progesterone, estradiol-17β and androgen in peripheral plasma of dairy cows during the first 60 days of pregnancy.

As the hormone levels were followed during the first 60 days of pregnancy on eight pregnant Holstein-Friesian cows by consecutive blood sampling every 3–5 days, we found that circulating androgen levels were maintained relatively constant during the first 60 days of pregnancy. Androstenedione and testosterone levels have been quantified in the peripheral plasma of dairy cows throughout pregnancy (Gaiani *et al.*, 1984). By measuring the androgen concentration with a total of 54 blood samples collected from 54 cows (each cow sampled once) during 10–270 days of gestation, Gaiani *et al.* (1984) reported a steady secreation patterns of androstenedione and testosterone in Holstein-Friesian cows during the first 90 days of pregnancy. In addition, Mostle *et al.* (1983) studied the changes in circulating concentrations of androstenedione, epitestosterone and testosterone, sampled at monthly-intervals, during the first 6 months of pregnancy in dairy cows and found that the mean concentrations of all three hormones remained fairly constant in the first 2 months of pregnancy but rose significantly at the 3rd month of pregnancy. Although the antiserum used in our androgen assay reacted primarily with testosterone (100%) and 5α-dihydrotestosterone (74%), the androgen levels during the first 60 days of gestation were very comparable to the levels of testosterone reported by Gaiani *et al.* (1984) but somewhat lower than those measured by Mostl *et al.* (1983).

In the present study, the patterns of circulating androgen levels during the first 60 days of pregnancy appeared to follow a similar pattern to that of estradiol-17β. A similar finding has been reported in cows during the estrous cycle (Shemesh and Hansel, 1974; Kanchev and Dobson, 1976). However, a previous study in our laboratory (Yu and Wang, 1983) demonstrated that circulating androgen levels remained in average of 300 pg/ml from 110 days through 10 days before parturition and then gradually decreased toward parturition. The pattern of a circulating androgen level during late gestational period was thus similar to the pattern of progesterone. Regarding the sources of the circulating androgen, evidences show that the corpus luteum, ovarian interstitial cells and follicular theca cells of cows are able to synthesize androgen *in vitro* (Mori, 1975; Shemesh *et al.*, 1975; Erickson and Magoffin, 1983; Ericksom *et al.*, 1985). The adrenal cortex is also a source of androgen. Kanchev *et al.* (1976) demonstrated a significant decrease in the circulating plasma level of testosterone as it occurred in betamethasone-treated adrenocortical suppressed heifers. Furthermore, placenta (Mongkopunya *et al.*, 1975) and fetal gonad (Shemesh and Hansel, 1983) have also been reported as sources of androgen production in cows during pregnancy. However, the relative contribution of these sources (ovary, adrenal contex, placenta and fetal gonad) to the peripheral pool of androgen during various gestational periods of cows is unknown.

Data from the literature concerning the secretion pattern of estradiol-17β in peripheral plasma of cows during early pregnancy were relatively variable with respect to the number of estradiol-17β peaks and the time at which the peaks exist. Such diversified results are likely due to different blood sampling frequency and assay systems employed. In a study following the sampling frequency interval of 2–12 days during the first 75 days of pregnancy, Wettmann and Hafs (1973) found decreases in estradiol-17β levels during the first 11 days of gestation and one peak was present around day 40 of pregnancy. With daily monitoring the levels of estradiol-17β during the first 30 days of pregnancy in cows, two transient peaks were observed respectively, between day 5 and 8 and between day 14 and 17 of pregnancy (Ford *et al.*, 1979). Only one peak around day 5 of pregnancy was detected by Lukaszewska and Hansel (1981) who quantified estradiol-17β levels daily from day 1 through day 18 of gestation in cows. In the present study with a blood sampling frequency of 3-day intervals during the first 30 days of pregnancy and then 5-day intervals for the next 30 days of pregnancy, we observed that in addition to the peak detected around estrus, the peripheral estradiol-17β concentrations remained fairly static from day 3 through day 60 of gestation. This qualitative pattern was very similar to the results reported by Eley *et al.* (1979) while investigating the estradiol-17β secretion pattern during days 27-70 of pregnancy in cows.

As observed in the present study, the plasma progesterone levels increased in a linear fashion during the first 15-days of pregnancy ($r=0.64$, $p<0.01$), remained at a plateau of about 5 ng/ml during 15-21 days of pregnancy and elevated further to a second plateau of 6-7 ng/ml during days 24-60 of the gestation period. The absolute levels of progesterone measured in our study were very much comparable to the corresponding values measured every 5 or 10 days during the first 60 days of pregnancy in cows by Donalson *et al.* (1970), but were somewhat higher than those reported by Ford *et al.* (1979) and were lower than those of others (Wettemann and Hafs, 1973; Lukaszewska and Hansel, 1980) during the early pregnancy of cows. In general, they all agreed that after day 20 pregnancy, the levels of progesterone remain steadily high, with values of 5-12 ng/ml plasma or serum, depending on experimental conditions and assay systems. However, the reported peripheral progesterone secretion patterns during the first 20 days of pregnancy are relatively variable among different laboratories. Some investigators (Donaldson *et al.*, 1970; Wettemann and Hafs, 1973; Lukaszewska and Hansel, 1980; Hansel, 1981) observed a progressively increasing linear fashion, while others (Glencross *et al.*, 1973; Ford *et al.*, 1979) detected a fluctuated pattern, during the first 20 days of gestation.

Maintenance of pregnancy requires the balanced secretion of estrogen and progesterone (Bazer and First, 1983), although the role androgen plays is unknown. As discussed previously the values of circulating gonadal steroid levels in cows during pregnancy reported from different laboratories were variable due primarily to the differing assay procedures and sampling frequency. Consequently, changes in the ratios of steroids may be useful to express the constancy or alteration of the hormonal secretion in relation to different stages of gestation. The corpora lutea develops from granulosal and thecal layers following ovulation. If no fertilization occurs, the corpora lutea starts to lyze around day 15 of the estrous cycle in cows and the progesterone secretion drastically decreases; if fertilization occurs, the corpora lutea continues to exist and function throughout most of the gestation period (Bazer and First, 1983). Consequently, the circulating gonadal steroid patterns during the first two weeks of both cyclic and pregnant cows are identical; and the difference of hormonal profiles initiates thereafter depending on whether the animal is fertilized or not. As indicated in Table 2, the progesterone/estradiol-17β ratios ranged from 28 to 1,060; the ratio increased drastically during the first 6 days, gradually increased until day 30, and reached a constant ratio afterwards. The progesterone/androgen ratio is qualitatively similar to that of progesterone/estradiol-17β. The changes in the ratios of progesterone/estradiol-17β

and progesterone/androgen largely reflect the changes in progesterone secretion during the early gestational period (0–60 days) as the circulating levels of both estradiol-17β and androgen were relatively constant except at estrus. The androgen/estradiol-17β ratio was the most constant ranging from 4.5 to 5.2 except at day 0 (estrus) with a smaller ratio due to peaked estradiol-17β values at estrus.

Studies of circulating androgen levels during the estrous cycle in cows have suggested that androgen may be only a precursor in the biosynthesis pathway of estrogen, may be involved in the regulation of follicular growth (Louvet *et al.*, 1975) or in the triggering mechanism for the onset of luteolysis (Kanchev *et al.*, 1976), and/or in the control of estrous behavior (Baird, 1977). In vitro study has shown that from day 13 to day 24 bovine blastocysts were capable of metabolizing neutral C steroids (androstedione, testosterone, etiocholanolone, etc.) to 5β-reduced steroids (Chenault, 1980). Consequently, these androgens and their metabolites may play roles in embryonic growth and development, heme biosynthesis in the embryonic blood forming tissues, uterine metabolism or the maternal recognition of pregnancy (Chenault, 1980). Cyclic changes in morphology and secretions of reproductive tract during the estrous cycle and the maintenance of subsequent gestation are regulated by the balanced secretion of estrogen and progesterone (Bazer and First, 1983). Testosterone is known to stimulate uterine growth in the rat (Rochefort *et al.*, 1972; Ruh *et al.*, 1975), and androstenedione involvement in the development of the glandular component of the endometrium during the menstrual cycle has also been postulated (Bonney *et al.*, 1984). Moreover, androgen has been known to stimulate protein synthesis in many organs under physiological conditions. Whether androgen plays a role in gestation, synergistically with estrogen and/or progesterone, requires further investigation.

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荷蘭種系乳牛懷孕前期六十天血中雄性素、雌二醇及助孕酮濃度與比例之變化型態

余玉林　廖錦佳　林慶雄　陳茂墻

本研究探討荷蘭種系乳牛懷孕前期六十天血中雄性素，雌二醇及助孕酮含量之變化。血液採自 8 隻 3～7 歲經產乳牛；以頸靜脈採血，懷孕前期 30 天，每隔 3 天採血一次；而 30～60 天期間，每隔 5 天採血一次。血漿經乙醚萃取後，以放射性免疫法定量雄性素，雌二醇，及助孕酮。在懷孕前期 60 天期間，雄性素濃度呈現相當恆定，在 25～41 pg/ml plasma。懷孕 3 天內，血漿雌二醇濃度從 13.1 pg/ml 急劇下降至 7 pg/ml；此後則維持在 6～8 pg/ml，非常恆定。因此，懷孕前期 60 天期間，血中雌二醇與雄性素濃度型態極相類似。助孕酮之濃度爲三種所測類固醇之最高者，在懷孕 15 天期間直線上升，在 15～21 天時，其濃度爲 5 ng/ml plasma，而在懷孕 24～60 天期間稍升高至 6～7 ng/ml plasma。

本研究發現乳牛懷孕前期 60 天血中雌二醇與雄性素濃度變化型態極爲吻合。雄性素，除是生成雌二醇之前質以外，是否在早期懷孕期間扮演任何角色，值得繼續探討。