Estrous Cycle and Follicular Growth in Shiba-Goats

シバヤギの性周期と卵胞発育

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東京大学大学院農学生命科学研究科獣医学専攻
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Preface

Preface

Puberty is an important reproductive phenomenon in the domestic animals. Many reports described about endocrine patterns, age and body weight associated with puberty(Arije and Wiltbank 1971, Gregory *et al.*, 1978, McCarthy *et al.*, 1979, Suttie *et al.*, 1991,). In the female, puberty is usually associated with the first appearance of estrus, but, puberty also includes the events that immediately precede the onset of ovarian activity. In domestic animals, the first mating period is also important in practical aspect.

The classical "gonadostat" theory for heifers suggests that hypothalamic synthesis of gonadotrophin-releasing hormone (GnRH) is very sensitive to negative feedback inhibition by estrogen (Kinder *et al.*, 1987). During this period, small amounts of estrogen are sufficient to inhibit the synthesis and release of GnRH (Kinder *et al.*, 1995). Under these physiological conditions, follicles are not able to develop to any significant extent. At prepubertal period, two events happen that allow the establishment of ovarian cyclically. First, there is a decrease in the sensitivity of the hypothalamus to estrogen feedback. Within the first few weeks of reaching a new level in estrogen sensitivity, the hypothalamus begins to initiate the release of small surges of LH and FSH from pituitary. Within a short period of time, the pituitary releases a large amount of LH. While this surge dose not cause ovulation, it dose cause significant

production of progesterone from the ovary which lasts for several days (Berardinelli *et al.*, 1979). This , in turn, is immediately followed by another surge release of LH that leads to ovulation .

A short luteal phase of 8-12 days precedes the first estrus at 10~11 to 14 months of age in heifers , and this was followed by normal length of luteal phase (Schams *et al.*, 1981; Dodson *et al.*, 1988). After the demise of the transient luteal structures, puberty is attained with occurrence of the first behavioral estrus that is accompanied by ovulation and development of a corpus luteum with a normal lifespan (Kinder *et al.*, 1995). Thus, puberty can be defined as the date when blood concentrations of progesterone first elevate and follow a trait of characteristic pattern lasting about 2 weeks (Dodson *et. al.*, 1988).

On the other hand, puberty usually is influenced by breed, sires, photoperiod and the level of nutrition. Sires significantly influence age and weight at puberty, and high preweaning growth rate and heavy weaning weight were associated with early puberty and heavy weight at puberty (Arije and Wiltbank, 1971).

Shiba-goats that have long been bred for meat consumption in Kyushu District of Japan. In 1968, some pairs of the goats were introduced to the Experimental Station for Bio-Animal Science of the University of Tokyo, Faculty of Agriculture, for the experimental model of ruminant species. Thereafter, they were successfully bred and increased in number and were distributed to many laboratories for many different research purposes. The

Shiba-goat is white colored, horned, and much smaller in size while sharing common characteristics with full size goats. Adult body weight is 15~22 kg in females and 24~28 kg in males (Kano *et al.*, 1977). The reproduction of this goat is characterized by its high fecundity (litter size=1.84) and complete lack of seasonality (Mori *et al.*, 1982; Mori *et al.*, 1983). But unfortunately, there was no report to describe the relationship between the gonadal steroid hormones with body weight from birth tracking to puberty.

In 1980, it was reported for the first time that the ultrasound imaging system or the real-time echography permitted a reliable early pregnancy diagnosis in mares (Plamer and Driancount 1980). This report triggered a development during the course of which sonography became an important tool for the management of reproductive problems. Since then sonography provided significant contribution to our better understanding of the early embryonic phase and has contributed significantly to new discoveries concerning the function of the uterus and ovaries. Today ultrasonography is applied in the reproductive and obstetrical examination of numerous other species. Savio *et al.*, (1988) , indicated that most estrous cycles in beef heifers are characterized by growth of 3 dominant follicles at different periods. In the goats, Ginther and Kot (1994) reported the observed number of growing 4-mm follicles per day differed from randomness, indicating that follicles, on the average, emerged in groups (waves). Averaged over all interovulatory intervals, the number of 3mm follicles on each day that later reached $\geqq$6 mm

followed a pattern of significant peaks on Day 0 (ovulation), 4 ,8 and 14. A follicular wave was defined by consecutive days of entry of follicles ≧6 mm into the wave, and the day of emergence was defined as the first day that the ≧6 mm follicles were 3 mm. According to Abeyawardene and Pope (1987), the maturation of the follicle is halted due to the negative feedback effect of increasing concentrations of progesterone on LH secretion. Therefore, during the normal estrous cycle, this first dominant follicle in the luteal phase dose not ovulate. It is important to determine the ovulatory capacity of this follicle because the variability of follicles to ovulate or to atrophy at different stages of the estrous cycle may influence how precisely different pharmacological methods can control the time of ovulation (Parfet *et al.*, 1989).

Based on the knowledge described above, this thesis is dealing with a project to induce incomplete (shorten) estrous cycles in Shiba-goats. In other words, the author tried to find a way how to induce ovulation in all the dominant follicles growing at different stages in an estrous cycle of the goat. This thesis consists of 3 chapters, and each chapter bears its own Abstract, Introduction, Materials and Methods, Results and Discussion sections. The references are listed altogether at the end of the thesis.

The experiments herein described consist of three steps as follows. In Chapter 1, the author studied steroid hormone changes associated with body weight and sexual maturation. Next, in Chapter 2, the author confirmed

that the dominant follicles of the first latent wave could be ovulated when the luteolysis was induced by PGF2α, and demonstrated that the exogenous progesterone treatment could nullify the effect of PGF2α on ovulation. Finally, in the Chapter 3, the author successfully induced in Shiba-goats of recurring ovulatory cycles at 5 to 7 days intervals, at least 4 cycles continuously. At the time of 4th induced-ovulation, a goat was exposed to a male. The female expressed normal estrous behavior, mated and became pregnant.

Chapter 1
Changes in Steroid Hormonal Levels during Sexual Maturation in Shiba-Goats

Changes in Steroid Hormonal Levels during Sexual Maturation in Shiba-Goats

Abstract

The changes in peripheral estradiol, progesterone (in females) and testosterone (in males) concentrations were monitored in Shiba-goats from birth to 50 weeks of age together with changes in body weight and onset of puberty in females.

Body weight at birth was 1.3 $\pm$ 0.1 kg (mean $\pm$S.E., n=5) in males and 1.2 $\pm$ 0.1 kg in females , and that at 50 weeks of age was 14.7 $\pm$ 3.9 kg in males and 12.3 $\pm$ 3.3 kg in females. Onset of puberty in females was well correlated with body weight at 50 weeks of age; in the heaviest animal (15.9 kg), the first ovulation and behavioral estrus was detected at 304 and 388 days, respectively and in the lightest animal (9.9 kg), at 395 and 477 days, respectively.

Progesterone was continuously at low levels from birth to 42 weeks of age in all females studied (n=5). The first evident elevation of progesterone concentrations lasting for about 2 weeks occurred in 2 of 5 females at 43, 45 weeks of age due to ovulation and following luteal formation. The rest of 3 goats did not show this elevation of progesterone levels until 50 weeks of age.

Estradiol levels were at extremely high levels on the day of birth. Then from 2 weeks to 50 weeks of age, the levels fluctuated between 2 to 10 pg/ml. The peak of estradiol concentration before ovulation was not detected by the blood sampling schedule in this study (once a week). Discernible follicular development in prepubertal goats between 4 and 18 weeks of age was observed by ultrasonography, suggesting follicular development would occur irregularly even before the onset of puberty or the first ovulation. Irregular elevations of estradiol levels in each animal would indicate irregular follicular growth which did not result in ovulation.

As a whole, testosterone levels in males gradually increased from birth to 50 weeks of age, associating large fluctuations. These fluctuations may be resulted from once a week sampling which may randomly hit a value between peak or nadir of pulsatile secretion of testosterone in these animals.

Introduction

The term estrous cycle refers to the rhythmic phenomenon observed in female of mammalian species (except some primates) in which there are regular but limited periods of sexual receptivity called estrus occurring at intervals that are characteristic for each species. The cycle length is defined as the time from the onset of one period of sexual receptivity to the next, or as the interval between two successive ovulations.

The term puberty is used to define the onset of reproductive life. In the female, puberty is defined as the initiation of cyclic ovarian activity usually associated with the first appearance of estrus, but puberty also includes the events that immediately precede the onset of ovarian activity.

The classical "gonadostat" theory suggests that the hypothalamic neuronal mechanism which regulates synthesis and release of gonadotropin-releasing hormone (GnRH) is highly sensitive to negative feedback inhibition by estrogen (Day *et al.*, 1984; Kinder *et al.*, 1987, Kinder *et al.*, 1995). During this time, small amount of estrogen is sufficient to inhibit GnRH synthesis and release (Ramirez and McCann 1963). Under these physiological conditions, it is believed that follicles are not able to develop to any significant extent. At prepubertal period two events happen that allow the establishment of ovarian cyclicity. First, there is a decrease in the sensitivity of the hypothalamus to estrogen feedback. Within the first few weeks after reaching a new level in estrogen sensitivity, the hypothalamo-pituitary axis

begins to initiate the release of small surges of LH and FSH. Within a short period of time, the hypothalamo-pituitary axis releases a large amount of LH. While this surge does not cause ovulation, it does cause significant production of progesterone from the ovary (Berardinelli *et al.*, 1979) which lasts for several days. This, in turn, is immediately followed by another surge release of LH that leads to ovulation.

It is reported (Dodson *et al.*,1988) that mean plasma LH concentrations decreased over a certain period of time from birth, largely due to a decrease in basal LH concentrations. Thereafter, mean plasma LH concentrations start to increase at a certain age depending on species and strain, mainly as a consequence of increasing frequency of episodic LH releases and their amplitude. Then finally, the hypothalamic GnRH releasing apparatus (Nishihara *et al.*, 1994) gains the capability to respond in a positive fashion to increasing estrogen concentrations and to release an amount of LH sufficient to initiate ovulation. The first ovulation is followed by a formation of functional corpus luteum which secretes high levels of progesterone for about 2 weeks (Berardinelli *et al.*, 1979). Thus, the onset of puberty could be monitored by detecting this elevation of peripheral progesterone concentration retrospectively. (Dodson, *et al.*, 1988; Dow *et al.*, 1982; Byerley *et al.*, 1987; D'Occhio *et al.*, 1988; Suttie *et al.*, 1991).

On the other hand, puberty usually is influenced by breed, photoperiod and level of nutrition. Nutritional status has an important influence on reproduction. The importance of body weight or body composition *per se*

has been pointed out in determining whether a female is capable of breeding for the first time (Frisch, 1988), and some investigators have tried to seek more-physiological mechanisms whereby information about body weight, body composition and nutritional status could be transmitted to the reproductive system (Foster *et al.*, 1985; Foster *et al.*, 1988; Cameron *et al.*, 1985). The body weight hypothesis considers that there is a critical body weight for puberty to occur, and that a mass of fat is critical (Frisch, 1988). In contrast, the 'metabolic hypothesis' considers that weight and fatness are consequences or correlates of metabolic changes occurring before and around the timing of puberty. A model to test the influences of level of nutrition on luteinizing hormone (LH) secretion has been developed using sheep (Foster *et al.*, 1985). Ovariectomized lambs are maintained at their weaning weight of ~20 kg by quantitative food restriction. Under these conditions, despite the lack of ovarian steroid negative feedback, the female is severely hypogonadotropic. There is little LH secretion, as evidenced by slow frequency pulses (Foster *et al.*, 1985,1989; Landefeld *et al.*,1989). These growth-restricted lambs respond within a few days to *ad libitum* nutrition by increasing the frequency of LH pulses (Foster *et al.*,1989).

The Shiba-goat is a Japanese native miniature goat; adult female and male weight is 15~22 kg and 24~28, respectively (Kano *et al.*, 1977). The reproduction of this goat is characterized by its high fecundity (usually twins or triplets) and complete lack of seasonality (Kano, *et al.*, 1977; Mori *et al.*, 1982). The present study has two aims to obtain the basic information for

future analysis of the mechanism of puberty in Shiba-goats; firstly, to study the association of the steroid hormone concentration changes with sexual maturation, and secondly, to determine body weight at puberty.

Materials and Methods

Animals and management

Puberty traits of Shiba-goats were evaluated under natural conditions. The 10 Shiba-goats used were 5 males and 5 females. They were born in August and September of 1994 at the Experimental Station for Bio-animal Science of the University of Tokyo, Faculty of Agriculture. Each goat was identified at birth and birth weight was recorded. Goats were weaned at 12-14 weeks of age and placed on a diet of 300 g alfalfa hay, 200 g beet pulp, 300 g hay, 100 g barley per head per day . Water were supplied *ad libitum*.

Female goats in this study had been monitored for expression of their estrous behavior every day, and were mated at the first detection of evident estrous behavior when exposed to an experienced sire. The day of this mating was recorded as the first mating.

Collection of blood samples

Blood samples (10 ml) were collected every once a week from the jugular vein between 0830 and 0930 hr, before feeding. After blood sampling, goats were weighed. The blood was allowed to clot, and the serum was separated and frozen at -20 ℃ until assayed for progesterone and estradiol concentrations in females and testosterone concentrations in males.

Hormones assay

Serum concentrations of progesterone, estradiol and testosterone were assayed in duplicate by enzyme immunoassay (EIA) using each of progesterone, estradiol and testosterone enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Protocols for each assay is presented below.

This assay is based on the competition between a steroid hormone and its labelled tracer linked to an acetylcholinesterase for a limited number of steroid hormone-specific rabbit antiserum binding sites. The concentration of the steroid hormone tracer is held constant, while the concentration of the free steroid hormones (derived from standard solutions or samples) varies. Thus, the amount of steroid hormone tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free steroid hormone in the well. The rabbit antiserum-steroid hormone (either free or tracer) complex binds to the mouse monoclonal anti-rabbit antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to acetylcholinesterase) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs the light at 412 nm wave length. The intensity of

this color, determined spectrophotometrically, is proportional to the amount of the steroid hormone tracer bound to the well, which is inversely proportional to the amount of free steroid hormone present in the well during the incubation.

EIA assay protocol for steroid hormones

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. Even freshly prepared Ellman's Reagent has some measurable absorbance, approximately 0.1 Absorbance Units (A.U.). The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity: Total enzymatic activity of the acetylcholinesteraselinked tracer.This is analogous to the specific activity of a radioactive tracer.

NSB (Non-specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antiserum a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antiserum can bind in the absence of free analyze.

%B/B0 (%Bound/Maximum Bound): ratio of the absorbance of a sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the B/B_0 values versus concentration of a series of well containing various known amounts of analyze.

Preparation (Store all buffer at 4 ℃)

EIA **Buffer Preparation:** Dilute the contents of one vial of EIA Buffer Concentrate with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated (NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.).

Wash Buffer preparation: Dilute the contents of the vial of Wash Buffer Concentrate to total volume of 5 liters with UltraPure water. Add 2.5 ml of Tween 20. (NOTE: Tween20 is a viscous liquid and cannot be measured by a pipet. A positive displacement device such as a syringe should be used to deliver small quantities accurately.) A smaller volume of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Tween 20 (0.5 ml/liter of Wash Buffer).

Sample Preparation: It is necessary that the sample be purified prior to the assay. In this study serum was extracted for progesterone, estradiol and testosterone assays.

Estradiol, Progesterone or Testosterone Acetylcholinesterase Tracer (Stable 2 weeks at 4 ℃): Reconstitute the Tracer with 30mℓ of EIA Buffer.

Estradiol, Progesterone or Testosterone Antiserum:
Reconstitute the Antiserum with 60mℓ of EIA Buffer.

Unfreeze Sample:

Estradiol,Progesterone or Testosterone Standard

Reconstitue the Estradiol,Progesterone or Testosterone standard with 1mℓ of EIA Buffer.

(The concentration of this solution will be 10 ng/mℓ.Store at 4℃ will be stable for approximately 6 weeks. But diluted standards stable only 1 day at 4℃.)

Aliquot 100μℓ of bulk standard (10 ng/mℓ) to S1

S1=100μℓ S0 + 900μℓ EIA Buffer ----------1000 pg/mℓ

S2 =500μℓ S1+ 500μℓ EIA Buffer ----------500 pg/mℓ

S3 =500μℓ S2+ 500μℓ EIA Buffer ----------250 pg/mℓ

S4 =500μℓ S3+ 500μℓ EIA Buffer ----------125 pg/mℓ

S5 =500μℓ S4+ 500μℓ EIA Buffer ----------62.5 pg/mℓ

S6 =500μℓ S5+ 500μℓ EIA Buffer ----------31.25 pg/mℓ

S7 =500μℓ S6+ 500μℓ EIA Buffer ----------15.62 pg/mℓ

S8 =500μℓ S7+ 500μℓ EIA Buffer ----------7.81 pg/mℓ

ASSAY

1. Plate Set Up (Record the contents of each well)

2. Rinse the plates with Wash Buffer and to remove the Buffer from the plate

3. Pipet the reagents($\mu\ell$)

4. Incubate the plate

Cover the plate with plastic film and incubate for one hour at room temperature.

5. Develop the plate

Empty the well and rinse five times with Wash buffer.

6. Unfreeze Ellman's Reagent

Unfreeze Ellman's Reagent and reconstitute the Ellman's Reagent with 50 ml Ultra pure water. Add 200 $\mu\ell$ of Ellman's Reagent to each well and 50 $\mu\ell$ of tracer to the Total Activity wells. Cover the plastic film. To develop the plate on the plate shaker and in the dark about 70-80 minutes.

7. Read the plate at 414 nm.

Read the plate with immunoMini NJ-2300 (SYSTEM INSTRUMENT Co., LTD JAPAN) and data were calculated by ELISA DATA analysis system: immunoPlan for WINDOWS V 1.0 .

Protocol for estradiol extraction and defatting

Preparation

1. Wash and Dry up the long tube with 0.5 ml of methanol and numbering.
2. Solution
   Methanol (WAKO)
   Diethyl Ether (WAKO)
   Hexane (WAKO)
   50% Methanol

Extraction

1. Sample 2 ml + Ether 6 ml Vortex
2. Repose
3. Snap freezing
4. Decanting
5. Dry up (Evaporator 42 ℃, blow with N_2)
6. Rinse with 0.5 ml Diethyl Ether
7. Dry up (Evaporator 42 ℃, blow with N_2)
8. 50% Methanol 0.5 ml + Hexane 1 ml
9. Vortex

10. Add 4 ml Hexane .
11. Centrifuge (4℃ 3000 rpm 5')
12. Aspirate
13. Add Hexane 2 ml
14. Repose 5'
15. Aspirate
16. Dry up (Evaporator 70 ℃, blow with N_2)
17. Rinse with 0.5 ml Hexane
18. Dry up (Evaporator 70 ℃, blow with N_2)
19. Fill up with N_2 and close up with parafilm
20. Put it in freezer (20 ℃) until assay.

Protocol of extraction of progesterone and testosterone

Preparation

1. Washing and Dry up the long tube with Methanol 0.5 ml and numbering.
2. Solution

Diethyl Ether (WAKO)

Extraction

1. Sample O.05 ml + Diethyl Ether 2 ml Vortex 5'
2. Repose 5'
3. Snap freezing 3'
4. Decanting
5. Dry up 40'(Evaporator 42 ℃, blow with N_2)
6. To rinse with 0.5 ml Diethyl Ether
7. Dry up (Evaporator 42 ℃, blow with N_2)
8. Fill up with N_2 and close up with parafilm
9. Put it in freezer (20 ℃) until assay.

Results

Birth weight in males averaged as 1.3 ± 0.1 kg and in females, 1.2 ± 0.1 kg. The weaning weight was 6.6 ± 1.2 kg in males and 5.6 ± 1.2 kg in females. Body weight of 50 weeks in male was 14.7 ± 3.9kg in males and 12.3 ± 3.3 kg in females. The age of the first mating was 441.6 ± 14.7 days of age (Table 1).

Changes in serum estradiol concentrations in females are presented in Fig. 1.1 together with changes in body weights. Estradiol levels were at extremely high levels at the day of birth. Then from 2 to 50 weeks of age, the levels fluctuated between 2 to 10 pg/ml. The peak of estradiol concentration before ovulation was not detected by the blood sampling schedule in this study (once a week). Discernible follicular development was observed in two goats by ultrasonography which were 4 and 18 weeks of age (Fig. 1.5).

Change in progesterone concentrations in females are presented in Fig. 1.2 together with changes in body weights. Progesterone was continuously at low levels from birth to 42 weeks of age in 5 of 5 females. The first evident elevation of progesterone concentrations lasting for about 2 weeks occurred in 2 of 5 females at 43, 45 weeks of age when body weights were 16.8 and 15.0 kg, respectively. Their first mating ages were 54.5 and 56.4 weeks when their body weights were 18.0 and 16.3 kg, respectively. The rest of 3 goats did not show this elevation of progesterone levels until 50 weeks of age. Body weights of them at 50 weeks of age were 11.8, 10.5, 7.5 kg and their

first matings were 59.3, 70.6, and 74.6 weeks of age when their body weights were 14.7, 12.2 and 11.0 kg, respectively. The body weight and age at the first mating was negatively correlated (Fig. 1.3).

As a whole, testosterone levels in males gradually increased from birth to 50 weeks of age (Fig. 1.4), associating with large fluctuations. Testosterone concentrations higher than 5 ng/ml could be observed only in the 3 goats with relatively heavier body weight until 50 weeks of age.

Discussion

Changes in body weight and peripheral steroid hormones level were monitored in both sexes from birth to 50 weeks of age. The first ovulation in females occurred earlier in animals with larger body weight and 3 of 5 females with smaller body weight in this study did not ovulate until 50 weeks of age. The age of the first mating was determined by detection of the first evident estrous behavior, and was negatively correlated with body weight. Arije and Wiltbank (1971) reported that heifers fed with a high nutritional level grew faster and reached puberty at younger age, and that body weight among heifers at puberty was considerably standardized, because animals with smaller body weight reached puberty at older age. Although this was also the case in this study, body weight at the first mating was still much variable. Body weight of the first mating in smaller goats was much smaller than larger goats, indicating that the body weight is not the direct determinant of onset of puberty, although it has substantial influence.

Serum estradiol concentrations were extraordinary high at the day of birth then decline to variable levels between 2 to 10 pg /ml from the 2 to 50 weeks of age. The initial high levels of estradiol in female neonatal rats is ascribed to the presence of fetus specific serum protein α-feto protein which binds to estradiol with high affinity (Barbanel and Assenmacher 1980). The exact reason for high estrogen levels in neonatal goats will be an object of future study. Variation in estradiol levels during prepubertal

period was also observed in goats by Venturina and Obsioma (1994) and in heifers by Adams *et al.* ,(1994) who suggested that follicles would start to grow and this growing wave would recur during prepubertal period. Two female goats at 4 and 16 weeks of age in this study were found to have middle to large sized follicles in their ovary by ultrasonography (Fig. 1.5), confirming their suggestion. Thus in goats, follicular development would occur irregularly from very early age. Irregular elevations of estradiol levels in each animal would indicate irregular follicular growth which did not result in ovulation.

This follicular growth should be brought about by gonadotropin stimulation. If so, the hypothalamo-pituitary-gonadal axis in goats seems to be active form very early age, because sporadic elevations of estradiol concentrations were observed from 4 weeks of age. This feature is in contrast with human or primate species where gonadal activity is completely suppressed for a certain period of time before puberty. Some essential mechanisms of the onset of puberty might be different between ruminant and primate species, which will be an object of future study.

Progesterone in female goats stayed continuously at precipitous levels before an abrupt increase occurred later than 40 weeks of age. Significant increases in progesterone concentration occurred in 2 of the 5 goats, the first and the second largest goats, at 43 and 45 weeks of age. The elevated levels could be observed in one or two successive samples and recurred at about 2 weeks interval. Thus, these elevated levels of progesterone concentration

can be attributable to ovulation and following formation of the functional corpus luteum. The rest of 3 goats did not show this elevation of progesterone levels until 50 weeks of age and the timing of the first ovulation could not be decided in this study.

It is believed that progesterone increase is required beforehand for ruminant species to express complete estrous behavior (Gonzalez *et al.*, 1975). This pattern seems to be common to the resumption of estrous cycles after periods of anovulation, for example, in beef heifer at puberty, cows after parturition and sheep after seasonal anoestrus (Thorburn *et al.*, 1969). Progesterone for this role is supplied from either granulosa cells in matured follicles under precedent stimulation of small release of LH and FSH, or functional corpus luteum formed after ovulation. In this study, an interval between the first ovulation and first mating could be assessed in the largest two goats as approximately 10 weeks. This 10 weeks period can allow goats to recur 3 to 4 estrous (ovulatory) cycles. The first mating in this study is based on detection of the first evident estrous behavior observed by an experienced animal care taker. Probably, the first estrous behavior would have been detected much earlier by use of a teasing male. It can be said from this study that expression of estrous behavior is gradually intensified by post-pubertal young goats recurring a couple of ovulatory cycles.

As a whole, testosterone levels in male goats gradually increased from birth to 50 weeks of age, associating with large fluctuations. These fluctuations may be resulted from once a week sampling which randomly

hits a value between the peak and the nadir of pulsatile secretion of testosterone in these animals. Again as discussed in female goats for their changes in estradiol concentrations, the hypothalamo-pituitary-gonadal axis of male goats seems functional at their early life. Secchiari *et al.*,(1976) reported that testosterone levels in male heifers increased gradually between 4 and 5.5 months, and oscillated between 2 and 4 ng/ml at intervals of about 2.5 month after 6.5 months of age before the onset of puberty (successful mating). The trend of testosterone secretion during sexual maturation is similar between goats and heifers.

The peak values exceeding 5 ng/ml appeared in 3 of the 5 males later than 34 weeks with larger body weight, but not in the rest of 2 goats with smaller body weight until 50 weeks of age. Endocrine maturity in male goats will be substantially influenced by their body weight as in female goats.

In summary, the present experiments support the hypothesis that follicular development occurs in prepubertal goat and estradiol concentration was variable with relatively high levels from 2 to 10 pg/ml. The concentrations of progesterone and testosterone developed a tendency to correlate with body weight.

Table 1.1 The age and body weight at first progesterone increase and first mating in female

Goat No.	Age at first progesterone increase (weeks)	Body weight 1 (kg)	Body weight 2(kg)	Body weight 3 (kg)	Body weight 4 (kg)	Age at first mating (weeks)
235	43	1.3	15.1	18.0	16.8	54.5
234	45	1.2	13.8	16.3	15.0	56.4
228	×	1.2		11.8	14.7	59.3
221	×	1.2		10.5	12.2	70.6
230	×	1.3		7.5	11.0	74.6

×: Had not an increase in progesterone concentration
Body weight 1: Body weight at birth
Body weight 2: Body weight at the first progesterone increase
Body weight 3: Body weight at 50 weeks of age
Body weight 4: Body weight at first mating

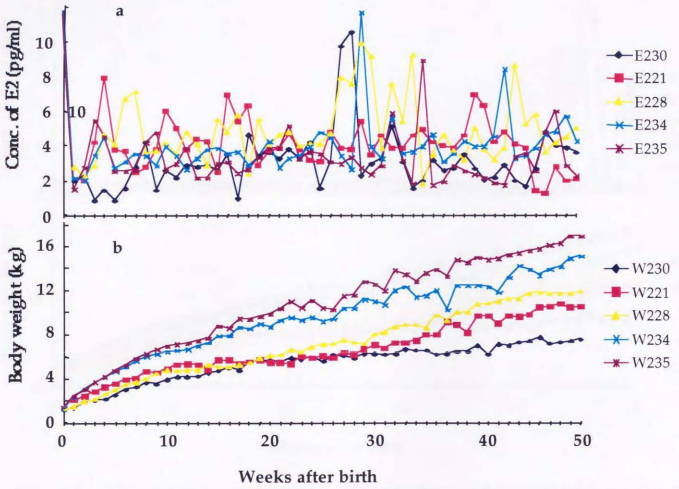


Fig.1.1 Changes in serum estradiol concentrations (a) and body weight (b) from birth to 50 weeks of age in 5 female Shiba-goats.

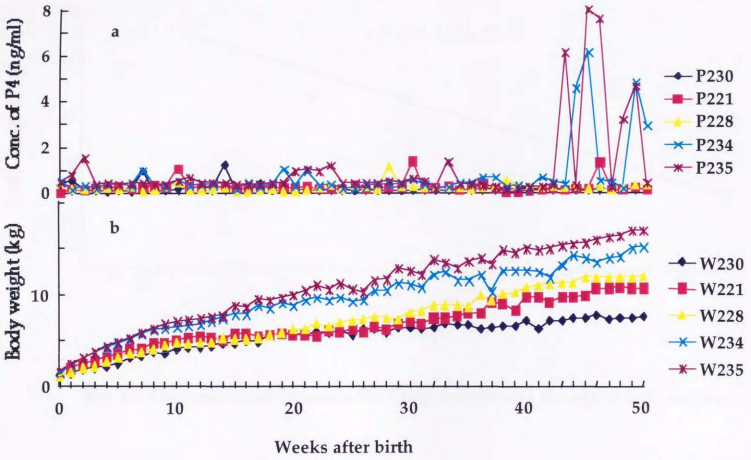


Fig. 1.2. Changes in serum progesterone concentrations (a) and of body weights (b) from birth to 50 weeks of age in 5 femaleShiba-goats.

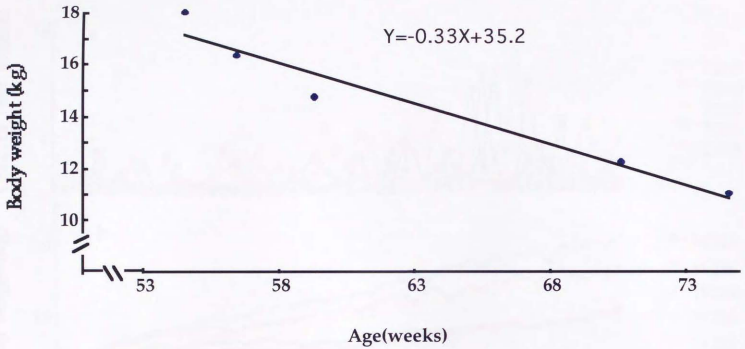


Fig. 1.3 The correlation between the body weight and the age at first mating .

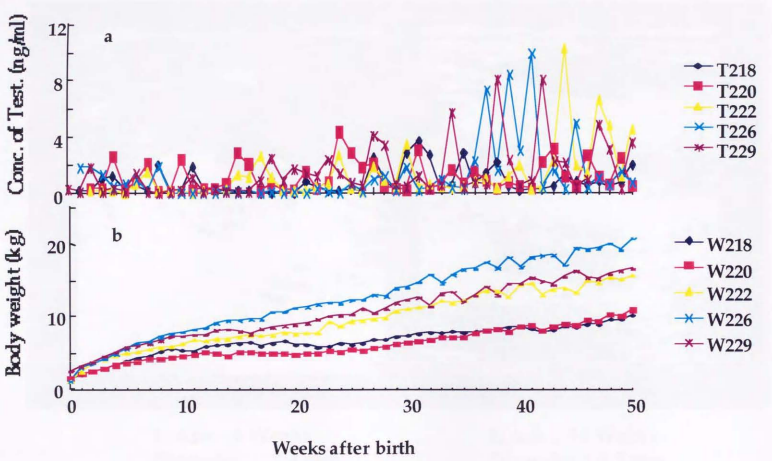
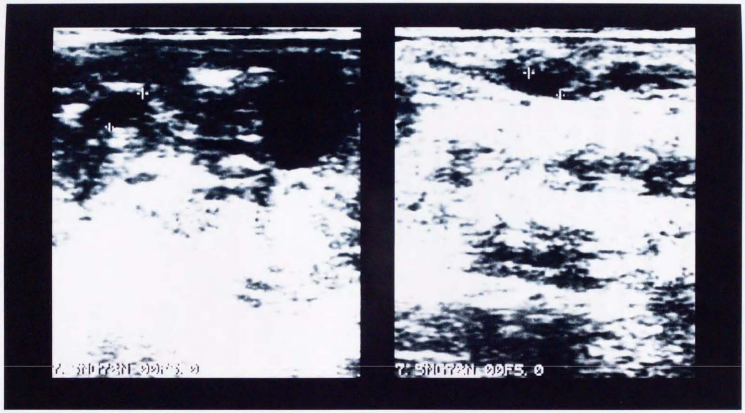


Fig. 1.4 Changes in serum testosterone cocentrations (a) and body weight (b) from birth to 50 weeks of age in 5 male Shiba-goats.



1. Age : 4 Weeks
Diameter : 5.4 mm

2. Age : 16 Weeks
Diameter : 4.7 mm

Fig. 1.5 Trans-rectum ultrasound observations of follicles in a Shiba-goat at the age of 1-(left panel) and 4- month(right panel).

Chapter 2
Follicular Waves during Estrous Cycle of Shiba-goats and Induction of Ovulation during Luteal Phase by Prostaglandin $F_{2\alpha}$

Follicular Waves during Estrous Cycle of Shiba-goats and Induction of Ovulation during Luteal Phase by Prostaglandin $F_{2\alpha}$

Abstract

Ovarian follicular development was studied in 12 female Shiba-goats by daily ultrasonographic examination during 2 successive estrous cycles. Two events of follicular growth not resulting in ovulation (latent follicular waves) occurred in 19 of the total 24 estrous cycles (79.2 %) during luteal phase, and one latent follicular wave was observed in 5 of the 24 estrous cycles (20.8 %). Thus, the estrous cycle in our herd of Shiba-goats consists of one ovulatory follicular wave and one or two latent follicular waves. The number of the ovulatory follicles and their diameter were 2.3 ± 0.9 and 6.9 ± 1.1 mm, respectively.

The estrous cycles consist of one ovulatory and two or one latent follicular waves, the mean length of which was 20.8 ± 1.7 and 20.3 ± 1.0 days (Mean $\pm$ S.E., n1=19, n2=5), respectively. And the dominant follicles derived from a cohort of the first latent follicular wave could be identified on Day 1 ± 0.3 and 1 ± 0.8 (day of ovulation=Day 0), reached maximum size (8.6 ± 1.6 and 11 ± 2.5 mm) on Day 4 ± 1.1 and 4 ± 0.5, began to decrease in size and became undetectable by Day 12.7 ± 1.6 and 15.1 ± 2.6, respectively. The dominant follicles derived from a cohort of the second latent follicular wave (not applicable to the estrous cycle with only one latent follicular wave)

could be identified by Day 7 $\pm$1.8(5 and 9), reached maximum size (6.6$\pm$0.9 mm) by Day 11.6$\pm$1.1 and became undetectable on Day 17$\pm$1.2. The dominant follicles derived from a cohort of the third follicular wave or the second ovulatory follicular wave (i.e., the next ovulatory follicular wave) were identified by Day 15 $\pm$2.7 and 14$\pm$2.3, reached maximum size (7.2 $\pm$ 0.9 and 6.4$\pm$0.7 mm) on Day 20.8$\pm$1.7 and 20.3,respectively, and ovulated.

Goats were treated with a luteolytic dose of prostaglandin (PG) $F_{2\alpha}$ on Days 3 (4 mg) and 4 (2 mg), when the dominant follicles of first latent wave were expected to be in growing phase and diameter were over 5.5 mm. Ovulation occurred from the dominant follicles of first latent wave. And this treatment significantly decreased the concentration of progesterone and increased the concentration of estradiol. Simultaneous progesterone treatment (60 and 30 mg) with $PGF_{2\alpha}$ inhibited the occurrence of ovulation and an associated elevation of plasma estradiol level. These results indicate that the growing dominant follicles derived from a cohort of the first latent ovulatory wave can ovulate spontaneously, if peripheral progesterone levels are depleted.

Introduction

Folliculogenesis involves the recruitment of a cohort of primordial follicles to enter the growing pool. A small number of these recruited follicles are selected to continue to grow, and finally, the development of dominant follicles which continue to grow suppresses the growth of other follicles (Hodgen,1982; Goodman and Hodgen, 1983; Fortune *et al.*,1991; Sunderland *et al.*,1994; Kaneko H., 1995). The dominant follicle is usually the largest follicle which has higher concentration of estradiol than either progesterone or androgens in the follicular fluid, and it is the source of most of the estradiol released into the blood stream (Ireland and Roche, 1982; Ireland and Roche 1983a).

Since Rajakski (1960) reported two 'waves' of follicular growth during an estrous cycle of cattle, one between Day 3 and 12 (latent follicular wave) and the other between Day 12 and the subsequent estrus (ovulatory follicular wave), numerous studies have been carried out to assess the dynamics of follicular development. There were some debates about the presence of latent follicular waves in cattle for a while; Donaldson and Hansel (1968) reported that growth and atresia of ovarian follicles appeared to be a continuous process, and no distinct mid-cycle waves in follicular growth were identified. However, it is now a general understanding that there are one or two latent follicular waves in cattle; Matton *et al.*, (1981) reported that the growth and replacement of large follicles was more rapid at the end

than at the beginning of the estrous cycle and that there were periods of growth of follicles between Day 3 and 13, Day 13 and 18, and Day 18 and the day of estrus. Ireland and Roche (1983b) reported similar results.

Until several years ago, the studies of ovarian follicular dynamics in large animals were limited to observations of ovaries at slaughter, ovariectomy, or surgery by marking follicles with such as India ink. These findings were limited either to a observation at a single time on each animal or to a limited number of observations in the same animal at surgeries. Therefore, it is difficult to obtain an accurate profile of the dynamics of follicular growth and regression throughout a complete estrous cycle.

Ultrasonography has been proved to be an effective means of monitoring and evaluating ovarian follicles and sequential changes in the size of individual follicles throughout an estrous cycle. After introduction of ultrasonography for monitoring follicular growth dynamics, morphological changes in patterns of growth and disappearance of dominant follicles has been studied in many species. Savio *et al.*, (1988) reported that most estrous cycles in beef heifers are characterized by growth of 3 dominant follicles at different times. This finding of sequential growth of 3 dominant follicles may help to explain the findings of previous workers describing ovarian follicular dynamics in cattle using methods other than ultrasound (Matton *et al.*, 1981; Ireland and Roche, 1982).

Ravindra *et al.*, (1994) described, the two phases of follicle emergence observed in ewes. However, the significant changes in numbers of antral

follicles and emergence of follicles associated with waves of follicular growth in cattle were not seen in the ewe. The follicular dominance associated with follicular growth waves in cattle was also not prominent in the ewe. Similar case also occurred in the goat; Ginther and Kot (1994) reported that the number of 4 mm follicles per day identified to be growing differed from randomness, indicating that follicles, on the average, emerged in groups (waves). They further reported that changes in the number of 3 mm follicles on each day that later reached ≧6 mm followed a pattern of significant peaks on Day 0 (ovulation), 4 ,8 and 14, one follicular wave was defined by consecutive days of entry of follicles ≧6 mm into the wave, and that the day of emergence was defined as the first day when 3 mm follicles later reached ≧6 mm appeared.

Similar results have been reported in other species.Pierson and Ginther (1987) and Ginther and Bergfelt (1993) reported about horses, Adams *et al.*, (1990) about llamas and Skidmore *et al.*, (1996) about camels, Douglas *et al.*, (1994) about minks. These reports support that "two or three waves hypothesis" in follicular development in ruminants and horses.

In spontaneous ovulators, ovulation occurs at the proper stage of the estrous cycle following normal follicular growth (Fortune, 1994). This means that a proper secretary pattern of estrogen is almost always able to elicit a surge release of LH that induces ovulation. Examples of spontaneous ovulators include the doe(goat), mare, cow, primates, and some laboratory species including the mouse, rat and,guinea pig. The dominant follicle(s)

that ovulates is the one(s) that develops concomitantly with the regression of the corpus luteum (Ginther *et al.*, 1989a). As mentioned above, follicular development during the estrus cycle in cattle had one or two dominant non-ovulatory follicles prior to the development of the ovulatory follicle. According to Abeyawardene and Pope (1987) and Convey *et al.*,(1977), the maturation of the follicle is halted due to the negative feedback effects of increasing concentrations of progesterone on LH secretion. Therefore, the first dominant follicle dose not ovulate during the normal estrous cycle, but can ovulate by induction of luteolysis with PGF2α (Quirk *et al.*, 1986; Kastelic *et al.*, 1990). Cattle treated with $PGF_{2\alpha}$ during the mid-diestrus had a longer interval from the treatment to estrus than that treated during early or late diestrus (Stevenson *et al.*, 1984). The size and viability of a dominant follicle of the first wave at the time of induced luteolysis may affect the length of the interval from the treatment to ovulation. Savio *et al.*, (1990) reported that the administration of PGF2α on Day 7 of the estrous cycle in heifers removed the negative feedback effect of progesterone on gonadotrophin secretion and allowed the ovulation of the first dominant follicle that consistently present at that stage of the estrous cycle.

The objectives of the experiments in this chapter are to study; 1) do the first dominant follicles of the estrous cycle of the Shiba-goat ovulate following PGF2α induced luteolysis? 2) When is the appropriate timing for the treatment with PGF2α to induce ovulation in the first dominant follicles? 3) what is the relation of plasma concentration of estradiol and

progesterone and the occurrence of ovulation? 4) does the simultaneous treatment with progesterone with PGF2α inhibit the occurrence of ovulation?

Materials and Methods

Animals

Twelve adult (1.5 year-old) female Shiba-goats weighing 29.9± 2.7 kg (24-30 kg) were used. They had been confirmed to recur regular 20-21 days estrous cycles before the start of experiment. The bilateral ovaries were examined by ultrasonography for at least 2 normal estrous cycles. During the experiment, goats were fed with 300 g alfalfa hay, 200 g beet pulp, 300 g hay, 100 g barley per head per day. Water was supplied *ad libitum*.

Ultrasonographic observation of ovaries and treatments

An ultrasonographic machine (SSD-550, Aloka Co., Ltd, Tokyo, Japan) was equipped with a Transrectum Electronic Linear Probe (UST-660-7.5, 7.5 MHz, Aloka Co., Ltd, Tokyo, Japan). The ultrasonographic examination was done daily. Each ovary was separately located and the transducer inserted per rectum and moved over the surface of the ovary (Fig. 2.1). Follicles, like other fluid-filled structures, appeared as black (nonechogenic) areas. When necessary, the image was frozen on the screen and the size of the follicle was measured. After each examination, diagrams of the relative positions of the follicles and relationship to other ovarian structures were drawn to trace individual follicles everyday. Individual follicles with antral diameters≧3 mm can be also identified on successive days with certainty by videotaping each ultrasound examination and reviewing the tapes

sequentially.

Spontaneously occurring ovulation was determined ultrasonically by the disappearance of a large follicle(s) that presented on the previous day as described by Ginther (1988) (Fig. 2.2). After confirming the spontaneous ovulation, goats were treated with PGF2α on two successive days. The first day of the treatment was usually on Day 3 when the diameter of a follicle(s) exceeded the size of 5.5 mm. A single intramuscular injection of 4 mg PGF2α (Fuji Chemical Industries Ltd., Takaoka, Japan) and the second injection of 2 mg PGF2α on the next day were done. Using another estrous cycle, the repetitive PGF2α treatments were done on Days 1 and 2. Ultrasonic examinations were continued until the day of induced ovulation or the next estrus.

Together with PGF2α, 5 goats were treated intramuscularly with progesterone (Luteogen L Sankyo Co., Japan) in 60 mg for the first day and 40 mg for the second day. Ultrasonic examinations were done once daily.

Collection of blood samples and hormone assay

Blood samples (5 ml) were drawn by jugular venipuncture into heparinized tubes (Terumo Venoject II, Tokyo, Japan) and immediately centrifuged. Plasma was collected and stored frozen (-20 ℃) until assayed. Plasma progesterone and estradiol concentrations were determined in duplicate by enzyme immunoassay after extraction (EIA assay and extraction protocol were described in chapter 1). Plasma (50 μl) was

extracted with 2 ml diethyl ether and the ether extract was assayed for progesterone without chromatography. For estradiol, 2 ml plasma were extracted with 6 ml diethyl ether and the ether extract was redissolved in water and methanol (50:50 v/v) and washed with hexane. The water/methanol layer was evaporated and used for the assay.

The assays were done using respective enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, USA).

Results

The mean length of the total of 24 estrous cycles studied was 20.7±1.3 days(Mean±S. E.) with a range of 17-22 days. In 19 cycles (79.2%), two latent follicular waves were identified (Fig. 2.3) and in 5 cycles (20.8%), one latent follicular wave was identified (Fig. 2.4). There were no cycles without latent follicular wave. The mean diameter of the follicles (164 ovulatory follicles) which were confirmed to ovulate one day later was 6.9±1.1 mm (Table 2-1).

Estrous cycles with two latent follicular waves

The mean length of cycles with two latent follicular waves was 20.8± 1.7 days (Fig. 2.4 and Table 2.2). The dominant follicles of the first follicular wave were identified on Day 1±0.3 (Day 0=day of ovulation), reached their maximum diameter (8.6±1.6 mm) on Day 4.1±1.1 and detectable until Day 12.7±1.6 (range; Day 9-14)(Table 2.4). After rapid growth between Days 1 and 4, the size of the dominant follicles began to decrease.

The dominant follicles of the second latent follicular wave were identified on Day 7±1.8 (range; Day 7-9), reached their maximum diameter (6.6±0.9 mm) on Day 11.6±2.1. Finally the dominant follicles of the third follicular wave (ovulatory wave) were identified on Day 15.1±2.7 (range; Day 14-17), grew slowly for about 2 days, then grew rapidly, reached their maximum diameter (7.2±0.9 mm) on Day 20.8±1.7, and ovulated one day

later.

Estrous cycle with one latent follicular wave

The mean length of the cycles with one latent follicular wave, was 20.3 ±1 days(Fig. 2.5 and Table 2.3), which was not significantly different from those with two latent follicular waves. The dominant follicles of the latent follicular wave were identified on Day 1.0±0.8, and reached maximum diameter (11.0±2.5 mm) on Day 4.0±0.5 and detectable until Day 15.1±2.6 (Table 2.4). The dominant follicles of second follicular wave (ovulatory wave) were identified on Day 14±2.3 (range; Day 12-17), reached their maximum diameter (6.4±0.7 mm) on Day 20.3±1.0 and ovulated on the next day.

Changes in steroid hormone concentrations during an estrous cycle

Plasma progesterone and estradiol levels were monitored throughout an estrous cycle in two goats with two latent follicular waves, together with the follicular growth dynamics (Fig. 2.6a, Fig. 2.6b).

Plasma progesterone levels began to increase one day after ovulation (Day 1), attained at maximum plateau levels from Day 4 to 12, and then started to decrease. The minimum level was observed on Day 17, probably immediately before the LH surge. On Day 18 an elevation of progesterone level was observed, which would probably be coincident with the occurrence of the LH surge that stimulated progesterone secretion from granulosa cells

of ovulatory follicles (Fig. 2.6a).

Most evident estradiol elevation was observed immediately before ovulation of the dominant follicles derived from the 3rd follicular wave. Much smaller elevations of plasma estradiol concentrations were observed when the dominant follicles either derived from the 1st or 2nd latent follicular wave reached at their maximum diameters.

Induction of ovulation in the dominant follicles derived from the 1st latent follicular wave with PGF2α

Using 7 goats whose follicular growth dynamics had been monitored ultrasonographically, luteolysis was induced by duplicated PGF2α injections for 2 successive days. The first treatment started when the diameter of a follicle(s) of the first latent follicular wave exceeded the size of 5.5 mm. As shown in Figs. 2.7 (lower panel) and 2.8, the first PGF2α injection induced an immediate depletion of plasma progesterone concentration, and an inverse elevation of plasma estradiol concentration. As a consequence, all of the 6 goats thus treated ovulated 2 or 3 days after the last PGF2α injection (Table 2.5).

Repetitive PGF2α treatment during a different stage of the estrous cycle failed to induce ovulation (Fig. 2.9). PGF2α was injected on Days 1 and 2, where plasma progesterone concentrations were not precipitated but followed the normal trait (maximum levels ≈8 ng/ml), and estradiol levels were never elevated until Day 15. A unique feature of follicular growth

dynamics was observed in this case; two follicles grew rapidly immediately after ovulation or the PGF2α treatment suggesting revival of the follicles in the 3rd follicular wave of the antecedent estrous cycle that had not ovulated and once destined to regress. These two follicles regressed together with the follicles of the first wave of the next estrous cycle on Day 12 or 13. Normal-like 2 follicular waves were observed in this estrous cycle (complete data are not shown) and ovulation occurred on Day 21 as normally.

Effect of simultaneous progesterone treatment with $PGF_{2\alpha}$ on induction of ovulation

In all the 5 goats treated for two successive days with progesterone (60 and 40 mg) simultaneously with the PGF2α, ovulation was not induced. When the time of the occurrence of the next ovulation was taken into account, 2 types of responses were identified (Table 2.6). Two of the 5 goats ovulated with a shortened interval (#1, 2; shortened type), the rest of 3 goats ovulated with an approximately normal interval (#3, 4,5; normal type).

In the shortened type(Fig. 2.10), progesterone and PGF2α were treated on Days 3 and 4 (#1) or on Days 2 and 3, (#2). On Day 3 or 2, progesterone level was still not elevated yet, and an elevation of progesterone level on Day 4 or 3 can be attributed to the exogenous progesterone treatment, because the endogenous progesterone secreting source was obliterated by the PGF2α treatment. A precipitous decline of progesterone levels before

Day 7 could allow the second (latent) follicular wave to grow and ovulation would occur in dominant follicles of this cohort. Estradiol levels were variable between Days 7 and 12 or 11 (no further data), which may represent the process of follicular selection within this cohort.

In the normal-like type (#3, 4, 5)(Fig. 2.11), an elevation of peripheral progesterone level due to the exogenous treatment was blunted if compared to the shortened type, but progesterone levels were not precipitously decreased but maintained at ≈5 ng/ml on Day 7 and further, which may inhibit for the second (latent) follicular wave to grow. The following ovulation with a normal interval would be resulted from ovulations occurring in the dominant follicles of the third (ovulatory) follicular wave.

Discussion

From the present study it was confirmed that follicular growth dynamics in Shiba-goats can be monitored by daily ultrasonographic observation without giving goats much stress to disturb their estrous cyclicity. Technical problem was that it was difficult to pursue individual follicles accurately, when clear hierarchy of follicles had not been established and/or follicular diameters were less than 3 mm, as on Days 0 and 1 of the estrous cycle. Thus, majority of data dealt with follicles larger than 4 mm in diameter in this study.

The results of this study indicate that most estrous cycles in Shiba goats are characterized by growth of one or two latent follicular waves during luteal phase. This finding of sequential growth of one or two latent follicular waves in goats is similar to the reports in cattle (Savio *et al.*, 1988; Knopf *et al.*, 1989; Adams *et al.*, 1992a; Sunderland *et al.*,1996). But in Saanen goats, it was reported that there were three latent follicular waves (Ginther and Kot, 1994). Although the author classified the estrous cycle in Shiba-goats into two, detailed observation of the estrous cycles with one latent follicular wave strongly suggested the presence of the second latent follicular wave. The second latent follicular wave in this category is characterized by insufficient growth of follicles. The maximum diameter remained less than 4 mm, while that in the "two-latent-waves" exceeded 6 mm. Similar length of the estrous cycle between "two-latent-waves (20.

8±1.7 days)" and "one-latent-wave (20.3±1.0 days)" also suggests that the basic composition of these two cycles are the same.

Using goats with two latent follicular waves, plasma progesterone and estradiol levels were correlated with the follicular growth dynamics. Plasma progesterone levels began to increase one day after ovulation (Day 1), attained at maximum plateau levels from Day 6 to 15, and then started to decrease. This observation accorded with the observation in cattle by Ireland *et al.*, (1984). This elevation of peripheral progesterone is derived from the corpus luteum or corpora lutea which have formed by ovulation of the dominant follicle(s) of the third follicular wave(Rawlings *et al.*, 1984; Badinga *et al.*, 1992). The minimum level of progesterone was observed immediately before ovulatory LH surge, but an elevation of progesterone level soon followed. This transient elevation would probably be coincident with the occurrence of gonadotropin surge that stimulated progesterone secretion from granulosa cells of ovulatory follicles (Fig. 2.6). During the luteal phase when high progesterone levels were maintained, follicular growth seemed to be suppressed in general, but alternation of the cohorts of follicles occurred certainly as mentioned above.

Most evident estradiol elevation was observed immediately before ovulation of the dominant follicles derived from the 3rd follicular wave. This largest elevation of estradiol level will be responsible for induction of ovulatory gonadotropin surge or stimulation of GnRH surge generator in the hypothalamus. Much smaller elevations of plasma estradiol

concentrations were observed when the dominant follicles derived from either the 1st or 2nd latent follicular wave reached at their maximum diameters. These small elevation of estradiol levels correlated well with the growth of follicles of each 1st and 2nd latent follicular wave.

GnRH pulse generator activity determines basal gonadotropin levels which in turn, regulates growing rate of follicles. It is well known that GnRH pulse generator activity is suppressed by progesterone in decreasing its pulse frequency. Another aspect of gonadotropin secretion by the neural mechanism is that GnRH surge generator regulates the occurrence of ovulatory gonadotropin surge. GnRH surge generator is known to be stimulated by exposure to high levels of estradiol for a certain period of time and is inhibited by progesterone. Thus, the reason why the dominant follicles in the latent follicular wave do not ovulate is considered to be the presence of progesterone which is secreted from the corpus luteum or corpora lutea formed by ovulation in the antecedent third follicular wave. In fact, several authors (Abeyawardene and Pope, 1987; Gyawu *et al.*, 1991) previously suggested that the dominant follicles is prevented from final maturation by an increasing concentration of progesterone, which suppress LH secretion.

To confirm this hypothesis, goats were treated with PGF2α to induce luteolysis with the corpus luteum being present. The treatment was designed to induce luteolysis at the time when follicles in the first latent follicular wave grew enough to respond enhanced gonadotropin secretion

due to progesterone withdrawal. PGF2α injection induced an immediate depletion of plasma progesterone concentration, and an inverse elevation of plasma estradiol concentration, suggesting an increment of frequency of GnRH pulse generator. As a consequence, all of the 6 goats thus treated ovulated 2 or 3 days after PGF2α injection. This observation is consistent with the observation in cattle by Kastelic and Ginther (1989).

PGF2α treatment itself dose not seem to induce ovulation, because PGF 2α injected on Days 1 and 2 when the corpus luteum did not secrete significant amount of progesterone, could not cause ovulation. This inability can be interpreted that the corpus luteum at this stage is not responsive to PGF2α. The corpus luteum exposed to PGF2α treatment immediately after its formation followed the normal trait of progesterone secretory activity, indicating that the function of the corpus luteum is not affected by early PGF 2α exposure.

A unique feature of follicular growth dynamics was observed when the goat was treated with PGF2α on Days 1 and 2; two follicles grew rapidly from ≈4 to ≈8 mm between Day (-1) and Day 2. Thus, only the probable origin of these 2 follicles seems to be the cohort in the 3rd ovulatory follicular wave of the antecedent estrous cycle. If this is the case, PGF2α might have an effect to revive the follicles that had not ovulated and once destined to regress. These two follicles regressed together with the follicles of the first wave of the next estrous cycle on Day 12 or 13. The exact mechanism is the subject of future study.

If the ovulation-inducing effect of the PGF2α treatment is solely attributable to a depletion of peripheral progesterone levels due to its luteolytic action, simultaneous progesterone treatment with PGF2α should nullify the ovulation-inducing action of PGF2α. In all the 6 goats treated for two successive days with progesterone (40 and 20 mg) simultaneously with the PGF2α, induction of ovulation was blocked, verifying the hypothesis. When the time of the occurrence of the next ovulation was taken into account, 2 types of responses were identified. Two of the 5 goats ovulated with a shortened interval and 3 of the 5 goats ovulated with an approximately normal interval. The difference in these responses again seems to be attributable to peripheral progesterone levels after exogenous progesterone treatment. Because the endogenous progesterone secreting source was obliterated by the PGF2α treatment, progesterone levels in question can be attributed to the exogenous progesterone. Thus, the difference in declining rate of progesterone levels can be attributed to the difference in metabolic clearance rate of individual animals. Another possibility is a difference in progesterone secreting activity of the adrenal. If animals were under more stressful condition, progesterone can be supplied more from the adrenal. However, when animals were treated with only $PGF_{2\alpha}$, progesterone levels were always precipitously depleted. Thus, the two possibilities mentioned above do not seem to be the case. The author likes to study a possibility in future that a high levels of progesterone can somehow weaken the luteolytic effects of PGF2α.

In summary, the growing dominant follicles derived from a cohort of the first latent ovulatory wave can ovulate spontaneously, if peripheral progesterone levels are depleted.

The results of this chapter make the author to hypothesize that dominant follicles derived from all the follicular waves either latent or ovulatory can ovulate successively, if each precedent corpus luteum is obliterated by PGF2α.

Table 2.1. The length of estrous cycle and the number of ovulated follicles in one estrous cycle

Duration of estrous cycle	20.7 ± 1.3[a] days
Diameters of ovulatory follicules	6.9 ± 1.1 mm
Numbers of ovulated follicles in one estrous cycle	2.3 ± 0.9
The percentage of cycles having;	
One latent follicular wave	20.8 % (5/24)
Two latent follicular waves	79.2 % (19/24)

a: Mean ± S.E.M.

Table 2.2 Estrous cycles with two latent follicular waves

	First follicular wave	Second follicular wave	Third follicular wave
First identified day	1.0 ± 0.3	7.0 ± 1.8	15.1 ± 2.7
Day at maximum follicular size	4.1 ± 1.1	11.6 ± 2.1	20.8 ± 1.7
Detectable duration (days)	12.7 ± 1.6	17.0 ± 1.2	20.8 ± 1.7

Values are Mean ± S.E.
n=19
Day0 is defined as the day of ovulation.

Table 2.3 Estrous cycles with one latent follicular wave

	First follicular wave	Second follicular wave
First identified day	1.0 ± 0.8	14.2 ± 2.3
Day at maximum follicular size	4.0 ± 0.5	20.3 ± 1.0
Detectable duration (day s)	15.1 ± 2.6	20.3 ± 1.0

Values are Mean ± S.E.
n=5
Day0 is defined as the day of ovulation.

Table 2.4 The Maximum size of follicles in each wave

	First follicular wave	Second follicular wave	Third follicular wave
Two latent follicular waves	8.6 ± 1.6 mm	6.6 ± 0.9 mm	7.2 ± 0.9 mm
One latent follicular wave	11.0 ± 2.5 mm	6.4 ± 0.7 mm	

Values are Mean ± S.E.

Table 2.5 The reproductive response of Shiba-goat given prostaglandin$F_{2\alpha}$

No. of goat	Luteolysis	Failure of luteolysis	Interval between $PGF_{2\alpha}$ injection and ovulation
248	○		48 h
251	○		72 h
255	○		72 h
257	○		48 h
259	○		72 h
260	○		72 h
268		●	21 days

Table 2.6 The interval between the treatment with progesterone (P4) and prostaglandin F2α (PGF2α) and ovulation.

No. of Goats	Day of treatment with P4 & PGF2α	Day of ovulation	interval between the treatment and ovulation
1	Day 3 and 4	Day 16	13 Days
2	Day 4 and 5	Day 17	14 Days
3	Day 3 and 4	Day 20	16 Days
4	Day 5 and 6	Day 21	16 Days
5	Day 3 and 4	Day 22	22 Days

Day 0 is defined as the day of ovulation.

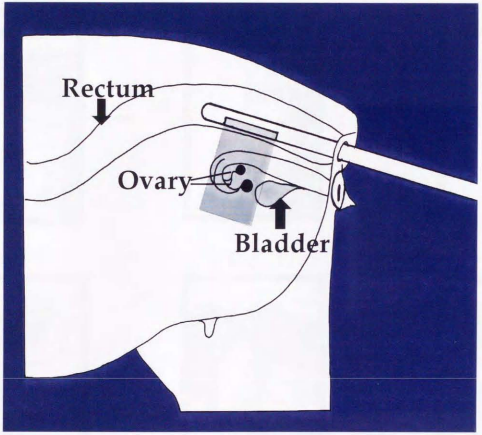


Fig. 2.1 Schematic presentation of the transrectal ultrasonography examination of the ovary in Shiba-goats. The probe is advanced about 15 cm into the rectum until the urinary bladder becomes visible. Follicles, like other fluid-filled structures, appear as black (nonechogenic) areas.

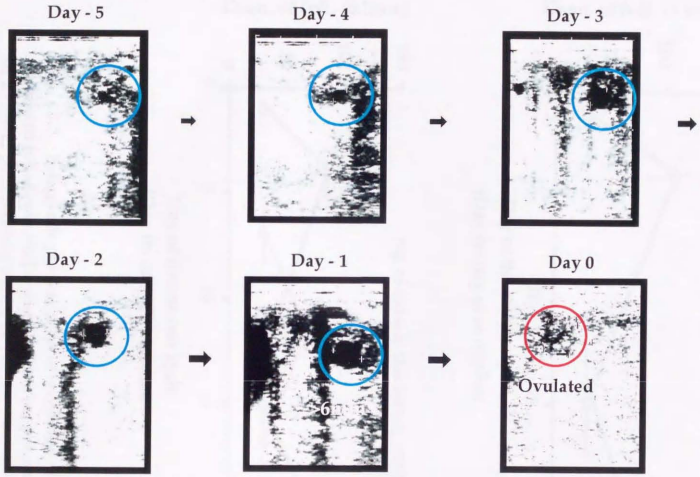


Fig. 2.2 Trans-rectum ultrasound observations of follicular growth in a Shiba-goat before ovulation (Day 0). When necessary, the image was frozen on the screen and the size of the follicle was measured. Ovulation was determined ultrasonically as the disappearance of a large follicle that was presented at previous examination.

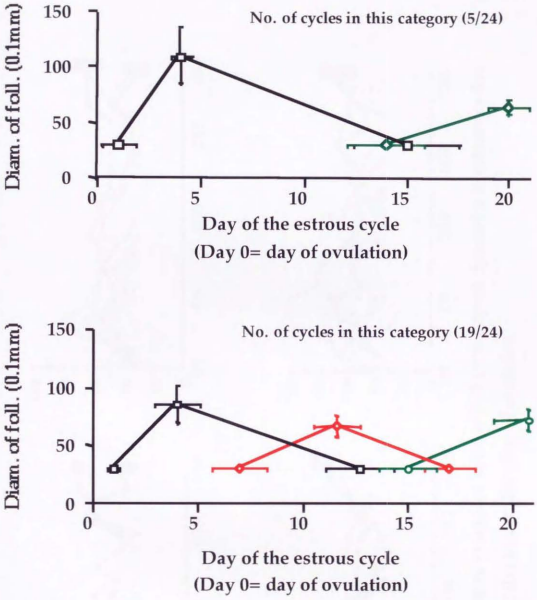


Fig. 2.3 Schematic presentation of dynamics of the dominant follicles in Shiba-goats with 1 (upper panel) or 2 (lower panel) latent follicular waves

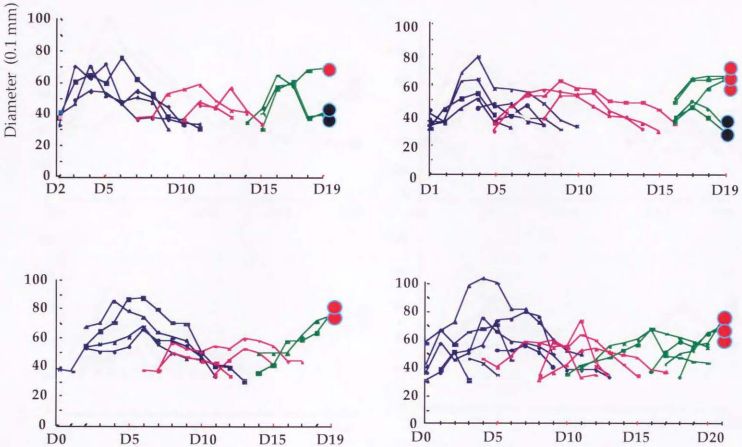


Fig. 2.4 Representative examples (4/19) of follicular growth dynamics in estrous cycles with two latent follicular waves. D0 : Day of ovulation

● Ovulated follicle
● Atretic follicle

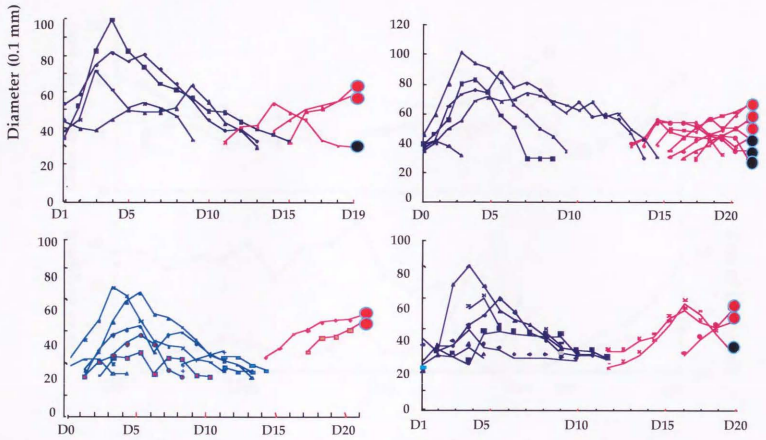


Fig. 2.5 Representative examples (4/5) of follicular growth dynamics in the estrous cycle with one latent follicular wave. D0: Day of ovulation.

- Ovulated follicles
- Atretic follicle

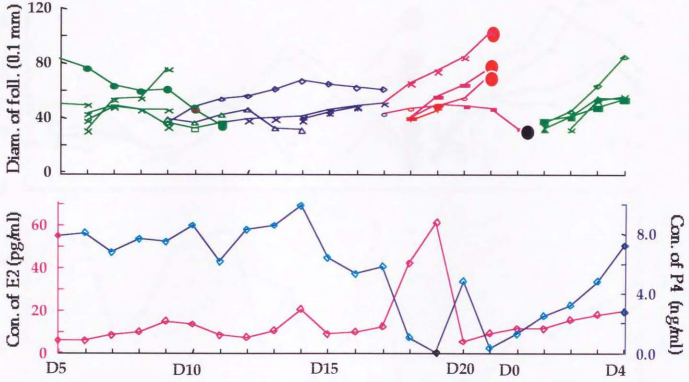


Fig. 2.6a Follicular development and steroid hormone concentrations during a normal estrous cycle in a Shiba-goat. D0: Day of Ovulation.
● Ovulated follicles ● Atretic follicle

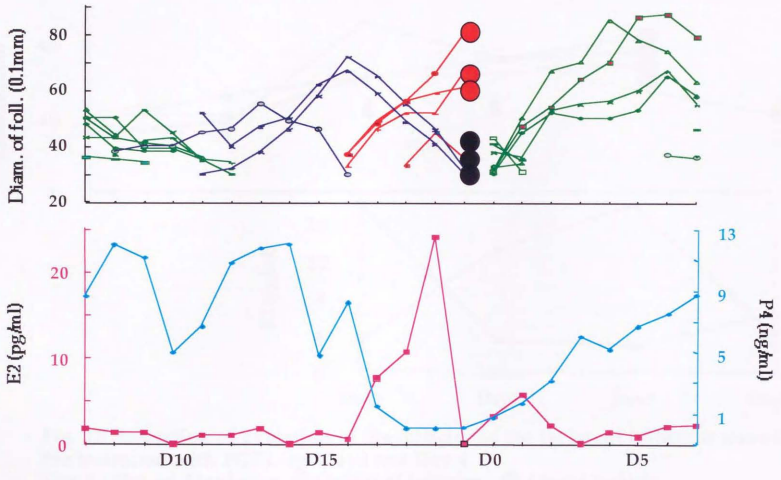


Fig. 2.6b Follicular development and steroid hormone concentrastions during a normal estrous cycle in a Shiba-goat. D0: Day of Ovulation
● Ovulated follicles ● Atretic follicles

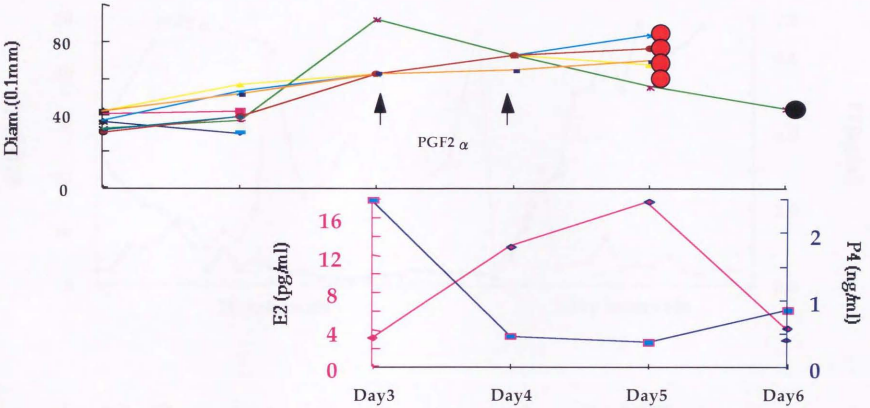


Fig. 2.7 Induction of ovulation of the follicles of the 1st latent follicular wave by the treatment with PGF2α on Day3 and Day 4.
Day 0 : Day of Ovulation. ● Ovulated follicles ● Atretic follicle

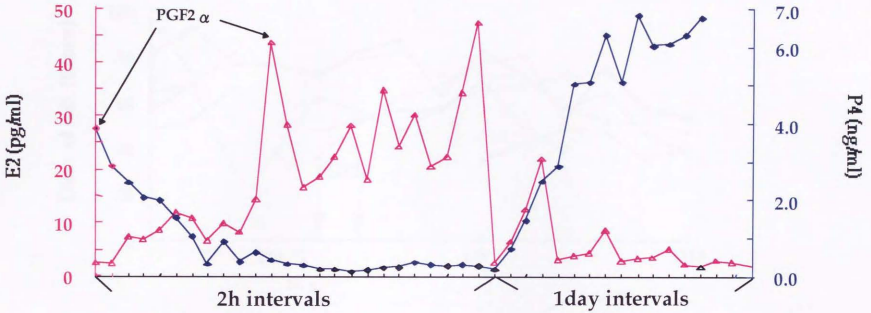


Fig. 2.8 Change in steroid hormone concentrations after PGF2α injection

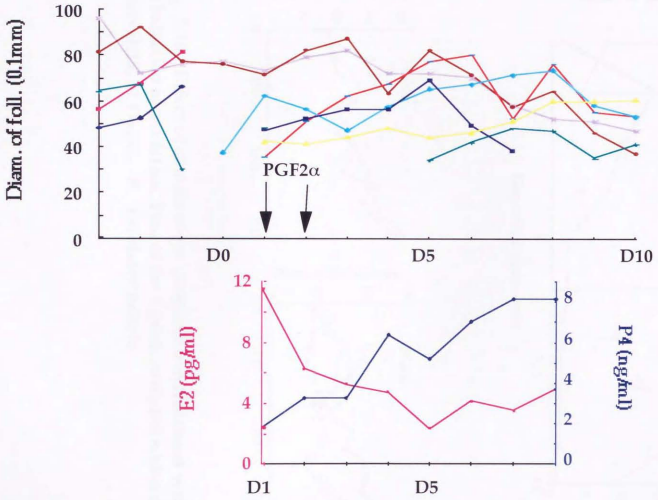


Fig. 2.9 Changes in follicular diameter and steroid hormone concentrations after the treatment with PGF2α on Day1 and 2.

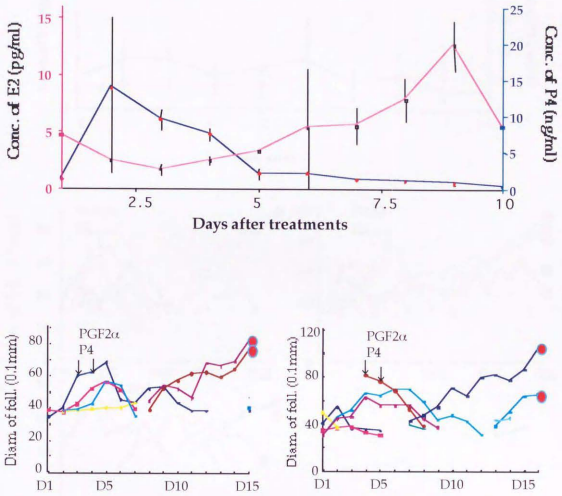


Fig. 2.10 Effect of simultaneous progesterone treatment with PGF2α on induction of ovulation. Two of the 5 goats ovulated with a shortened interval(16, 17 Days). ● Ovulated follicle

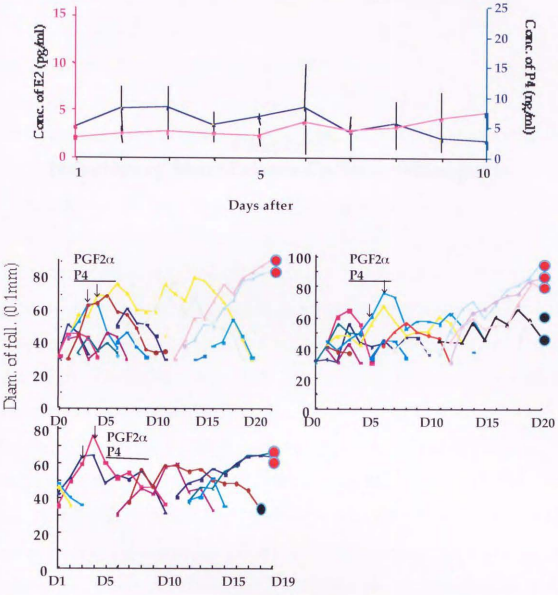


Fig. 2.11 Effect of simultaneous progesterone treatment with PGF2α on induction of ovulation. Three of the 5 goats ovulated with an approximately normal interval(20, 21 22 Days). ● **Ovulated follicle** ● **Atretic follicle**

Chapter 3
Induction of Short Estrous Cycles in Shiba-goats

Induction of Short Estrous Cycles in Shiba-goats

Abstract

To 6 matured female Shiba-goats, PGF2α was administered at every occasion when ultrasonographic observation detected that dominant follicles in consecutive follicular waves grew appropriately. According to this manner, all of the 6 goats were treated 4 times with PGF2α and ovulations were detected within 2 or 3 days after all of the treatments. Thus totally 24 ovulations were successfully induced. The mean length (±S.E.) of the 24 induced ovulation cycles was 6.5±1.2 days with a range of 5-9 days. Mean diameter of ovulated follicles is 7.0±1.1 mm and number of ovulated follicles per ovulation was 2.3±0.6.

Plasma progesterone concentrations had elevated to high levels between 4 to 12 ng/ml before the PGF2α treatment. The first PGF2α treatment, which was performed 2 or 3 days after the antecedent ovulation, induced precipitous decrease in these high progesterone levels. Then, rapid follicular growth was provoked and 1 to 5 follicles were selected as dominant follicles destined to ovulate. In concert with this progesterone depletion, estradiol concentration increased dramatically with one day delay, and then decreased to basal levels after the occurrence of ovulation.

One of the 6 goats was allowed to mate with a male after she repeated these short ovulatory cycles 4 times. She expressed normal estrous

behavior, successfully mated and was confirmed to be pregnant with one fetus by ultrasonographic examination.

These results suggest that the female goat is furnished with every necessary mechanism for recurring short estrous cycle (incomplete estrous cycle observed in rats and mice), if progesterone levels are kept nearly at nil.

Introduction

In the previous chapter, it is demonstrated that the administration of PGF2α which obliterates the functional corpus luteum secreting progesterone, results in removal of negative feedback effect of progesterone on gonadotrophin secretion, and allows the dominant follicle(s) of the first latent follicular wave to ovulate. Thus, the author tried to induce ovulations in each cohort of follicular waves in this chapter.

There are three main types of the estrous cycle seen in mammals. Type 1 is the incomplete estrous cycle, where short ovulatory cycles at 4-6 days' intervals recur in such animals as rats and mice. One estrous cycle consists mainly of follicular phase and progesterone secretory activity is virtually absent due to the induction of a progesterone catabolizing enzyme, 20α–hydroxysteroid dehydrogenase (20α-HSD) activity in the corpus luteum soon after its formation. Type 2 is the complete estrous cycle, where long ovulatory cycles at 16 or more days' intervals recur in many species of domestic animals (cattle, horse, goat, sheep, pig, etc.) and primates. One estrous cycle consists of about 7 days' follicular phase and about 14 days' luteal phase. Primates usually have an additional menstruation period lasting about 7 days. Type 3 is reflex ovulators, where copulatory stimulus is required to induce ovulation in such animals as ferrets, rabbits, cats etc. The estrous cycle halts at the estrous stage for a considerable period in the absence of copulation. Coitus is followed within 1/2~2 days by ovulation

and by pseudopregnancy, with an active corpus luteum of considerable duration(Swenson 1984).

In both spontaneous (types 1 and 2) and reflex ovulators, follicles rupture as the result of LH action with the difference that LH release in the former is cyclic, independent of copulation, and is provoked by positive feedback action of estrogen, whereas LH release in the latter occurs only when the proper stimulus, such as copulation or a facsimile of it, is applied to the cervix or parts of the vagina of the females.

In most female domestic animals (cow, horse, goat, sheep, and pig), the estrous cycle belongs to type 2. In this type, the corpus luteum is formed following ovulation under the influence of pituitary LH (luteotropic hormone) and secretes progesterone for a limited time (≈2 weeks in many species). Estrogen is produced in small quantities during the luteal phase except primates in which considerable amount of estradiol is secreted from the corpus luteum. The decline of the corpus luteum is associated with a decline in systemic blood levels of progesterone. There follows an increase in level of systemic estrogen, mainly estradiol-17β, which reaches a peak before the onset of estrus, and high level of circulating estrogen initiates the release of gonadotrophin, and rapidly induces release of FSH and LH by stimulating GnRH surge generator in the hypothalamus.

As was mentioned in previous chapter, follicular development during the estrous cycle in cattle (Pierson and Ginther, 1988), goat (Ginther and Kot, 1994), ewe (Ginther *et al.*, 1995) have one or two dominant nonovulatory

follicles prior to the development of the ovulatory follicle. According to the authors results in goats in the previous chapter, the final maturation of the follicle is halted due to the negative feedback effects of increasing concentrations of progesterone on LH secretion. Therefore, that first dominant follicle dose not ovulate during the normal estrus cycle. But results of the study in the previous chapter, Kastelic *et al.*, (1990), and Savio *et al* .,(1990) demonstrated that the dominant follicles of the first wave could ovulate when luteolysis is induced by PGF2α.

On the other hand, in the type 1 animals having the incomplete estrous cycle, the corpus luteum expresses 20α-HSD activity which catabolizes progesterone to a biologically inactive steroid 20α-dihydroprogesterone (20 α-OHP) (Lamprecht *et al.*, 1969; Smith *et al.*, 1975; Seong *et al.*, 1992). Exclusion of systemic progesterone by this way plays a crucial role for maintaining a unique short estrous cycle with 4 or 5 days' interval. Prolactin (PRL) suppresses 20α-HSD activity *in vivo* (Lamprecht *et al.*,1969) and *in vitro* (Matsuyama *et al.*, 1990). This inhibitory activity of PRL on 20α-HSD activity is a prerequisite for instituting pregnancy or pseudopregnancy. Indeed, the enzyme activity in the newly formed functional corpus luteum remains low and increases only at the end of pregnancy or pseudopregnancy (Matsuda *et al.*, 1990). This change in enzyme activity correlates well with the changes in peripheral progesterone and 20α-OHP levels (Saito *et al.*, 1988).

The study described in the previous chapter indicates that the goats

whose dominant follicles of the first latent follicular wave can be ovulated recover spontaneously the normal estrous cyclicity thereafter. This newly established estrous cycle should have again an escalated first latent follicular wave which once had been designated as the second latent follicular wave. Ovulation may possibly be induced again in the dominant follicles of this newly introduced first latent follicular wave. If this kind of sequence can be repeated, the goat will be able to repeat short (or incomplete) estrous cycles, and it may be said that the fundamental mechanisms for proceeding estrous cycles are common between types 1 and 2 animals, though the phenotype is much different from each other. Thus the objectives of the study in this chapter are: 1) Can ovulation recur every 5-7 days by induction of luteolysis repeatedly? 2) Can repeatedly induced ovulation be associated with normal fertility and/or estrous behavior?

Materials and Methods

Animals

The 6 female Shiba-goats used were 2 years old, weighed 29.9±2.7 kg and had shown regular 20-21 days estrous cycles at least twice before the start of the experiment. The goats were fed *ad libitum* before the start of experiment. During the experiment, goats placed on a diet of 300 g alfalfa haylage, 200 g beet pulp, 300 g hay, 100 g barley per head per day. Water was supplied *ad libitum*.

PGF2α treatment and ultrasonographic examination

Six mature female Shiba-goats were injected with PGF2α (Fuji Chemical Industries , Ltd., Takaoka, Japan) intramuscularly twice on 2 successive days (4 and 2 mg/goat) as described in chapter 2. The timing of the first treatment was decided ultrasonographically at every occasion when diameter of the dominant follicle(s) in a particular follicular wave became 5.5 mm or more. According to this manner, all of the 6 goats were treated 4 times, except one occasion where the PGF2α treatment was omitted.

Ovaries were examined once daily ultrasonographically as described in chapter 2.

One of the 6 goats was allowed to mate with an adult male after

confirming ultrasonographically the occurrence of ovulation 4 times by repeated $PGF_{2\alpha}$ treatments. Steroid hormone profiles were not analyzed in this goat.

Collection of blood samples and hormone assay

Blood samples (5 ml) were collected once a day throughout the experimental period from 5 of the 6 goats. Plasma progesterone and estradiol concentrations were determined by enzyme immunoassay as described in chapter 1.

Results

All the 6 goats were treated 4 times (with one exception where the 3rd PGF2α treatment was omitted) and ovulations were detected within 2 or 3 days after all of the treatments. Thus totally 24 ovulations were successfully induced (Figs. 3.1-3.6). The mean length (±S.E.) of the 24 induced ovulatory cycles was 6.5±1.2 days with a range of 5-9 days. Mean diameter of ovulated follicles is 7.0±1.1 mm and number of ovulated follicles per ovulation was 2.3±0.6 (Table 3.1).

Using 5 goats, plasma progesterone and estradiol levels were analyzed throughout the experimental period (Figs. 3.1-3.5). Plasma progesterone concentrations had elevated to high levels between 4 to 12 ng/ml before the PGF2α treatment. The first PGF2α treatment, which was performed 2 or 3 days after an antecedent spontaneous or PGF2α-induced ovulation, caused a precipitous decrease in progesterone levels. Then, rapid follicular growth was induced between Days 5 and 7, and 1 to 5 follicles were selected as dominant follicles destined to ovulate. In concert with this progesterone depletion, estradiol concentration increased dramatically with one day delay, and then decreased to basal levels after the occurrence of ovulation.

In one goat (#2) (Fig. 3.2), the 3rd PGF2α treatment was omitted, because follicular growth was so rapid and the goat expressed estrous behavior 2 days after ovulation. Although the 3rd PGF2α treatment was omitted, ovulation occurred spontaneously at a short interval of 4 days.

Plasma progesterone levels did not elevate between 2nd (induced) and 3rd (spontaneous) ovulations, suggesting a failure in formation of the corpus luteum after the second ovulation.

In another goat (#4)(Fig. 3.4), there seemed to be 7 estradiol peaks associating with 7 follicular waves within 29 days of an experimental period. However, there were only 4 progesterone peaks being coincident with the occurrence of ovulations. Thus, 3 follicular waves (marked with *) seemed to develop immediately before complete elevation of progesterone levels but did not result in ovulation.

One of the 6 goats (#6) was allowed to mate with a male after she repeated short ovulatory cycles 4 times. She expressed normal estrous behavior, successfully mated and was confirmed to be pregnant with one fetus by ultrasonographic examination (Fig. 3.7).

Discussion

The results of the present experiment clearly demonstrated that each of the repeated inductions of luteolysis with PGF2α allowed the formation of a follicular growth wave immediately after the precipitation of peripheral progesterone levels. The dominant follicle(s) in this newly escalated first follicular wave could again ovulate spontaneously by induction of luteolysis in the forerunning corpus luteum. After each induced ovulation, follicular growth started without any delay, and short estrous cycles recurred at 5-7 days intervals.

Follicular growth dynamics in a particular goat (#4) was fascinating. She expressed 7 follicular waves within 29 days of an experimental period, and the mean interval between the waves is calculated as 5 days which is similar to that in incomplete estrous animals such as rats or mice. Although ovulations could not be induced 7 times, the frequency of her follicular waves suggests that goat should have an innate capacity to recur follicular growth at a short interval. Progesterone secretion from the forerunning corpus luteum whould obscure this prototype feature of follicular growth dynamics in the goat.

Another fascinating example was observed in another goat (#2). The 3rd PGF2α treatment to her was omitted, because follicular growth was so rapid and she expressed estrous behavior 2 days after ovulation. Although the 3rd PGF2α treatment was omitted, surprisingly enough, ovulation

occurred spontaneously at a short interval of 4 days. Occurrence of this spontaneous ovulation can be ascribed to low plasma progesterone levels between the 2nd (induced) and the 3rd (spontaneous) ovulations. It may be still premature to narrate an exact reason for the absence of an elevation of progesterone level after the 2nd induced ovulation, but it seemed that the corpus luteum failed to acquire the function of progesterone secretion by some unknown reasons. If this was the case, the goat can form the corpus luteum deficient in progesterone secretory activity as rats and mice can. The collection of these cases and search for the true mechanism will be a subject of the future study.

It is well documented that 20α–hydroxysteroid dehydrogenase (20α-HSD), an enzyme which catabolizes progesterone to a biologically inactive steroid 20α-dihydroprogesterone (20α-OHP) plays a critical role in the maintenance of the unique 4 or 5 day estrous cycle by ultimately shortening the period of progesterone secretion. Sawada *et al* ., (1994) reported that progesterone and 20α-OHP were detected in blood following ovulation in goats. The changing pattern of both steroids in goats is different from that in rats; the fluctuations in 20α-OHP after ovulation are roughly in parallel with those in progesterone, though 20α-OHP levels are always lower than progesterone levels. After PGF2α treatment, a decrease in progesterone levels is again associated with a decrease in 20α-OHP levels. Thus, expression of 20α–HSD activity in goats (in the corpus luteum or other tissues?) is considered to be ubiquitous or constitutive, but not specific to the

stage of luteolysis as is so in rats. Stage and tissue specific expression of 20α–HSD gene in the rat should be regulated by the 5'regulatory sequence flanking to the gene. Mutation in this regulatory portion in the goat and probably in cattle may change the dynamics of 20α-OHP secretion, the function of 20α–HSD gene, and consequently the phenotypes of the estrous cycle from the incomplete to the complete one.

In the present study, the fertility of ovum shed after repetitive induction of ovulations was confirmed. Furthermore, the female goat expressed normal estrous behavior and could mate with a male after repetitive induction of ovulations. These results suggest that the female goat is furnished with every necessary mechanisms for recurring short estrous cycle (incomplete estrous cycle observed in rats and mice), if progesterone levels are kept nearly at nil. Fundamentals for constituting the estrous cycles look similar between the goat and the rat.

Table 3.1 The mean interval of ovulations and diameters of ovulated follicles and number of ovulated follicles

Interval of ovulations (day)	Diameters of follicles (mm)	number of ovulated follicles
6.5 ± 1.2 (n=24)	7.0 ± 1.1 (n=56)	2.3 ± 0.6 (n=24)

Values are Mean ± S.E.

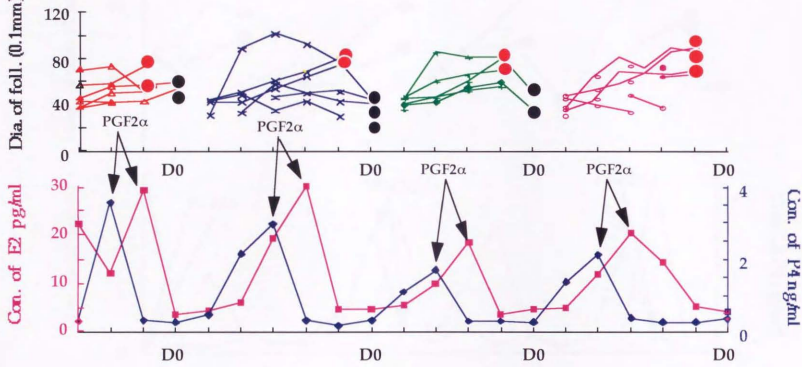


Fig.3.1 Induction of incomplete estrous cycles in a Shiba-goats #1. Changes in follicular diameter (upper panel) and serum progesterone and estradiol concentrations (lower panel). D0: Day of ovulation

● Ovulated follicles ● Atretic follicles

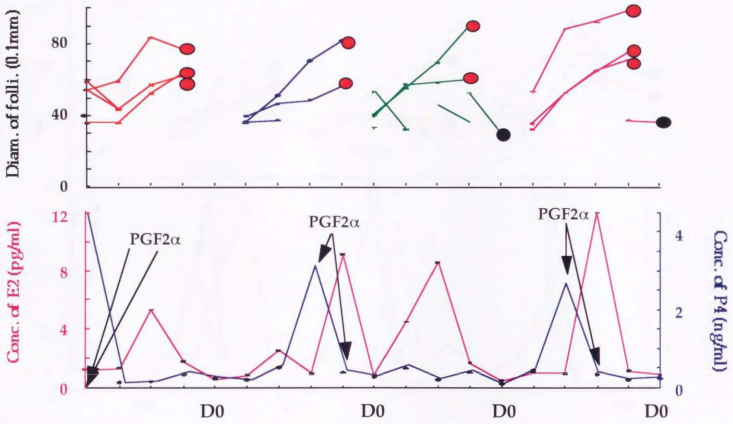


Fig.3.2 Induction of incomplete estrous cycles in a Shiba-goats #2. Changes in follicular diameter (upper panel) and serum progesterone and estradiol concentrations (lower panel). D0: Day of ovulation

● Ovulated follicles ● Atretic follicles

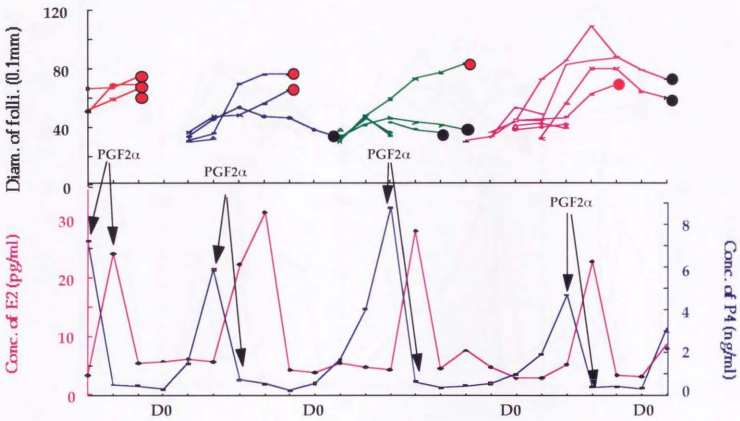


Fig.3.3 Induction of incomplete estrous cycles in a Shiba-goats #3. Changes in follicular diameter (upper panel) and serum progesterone and estradiol concentrations (lower panel). D0: Day of ovulation

● Ovulated follicles ● Atretic follicles

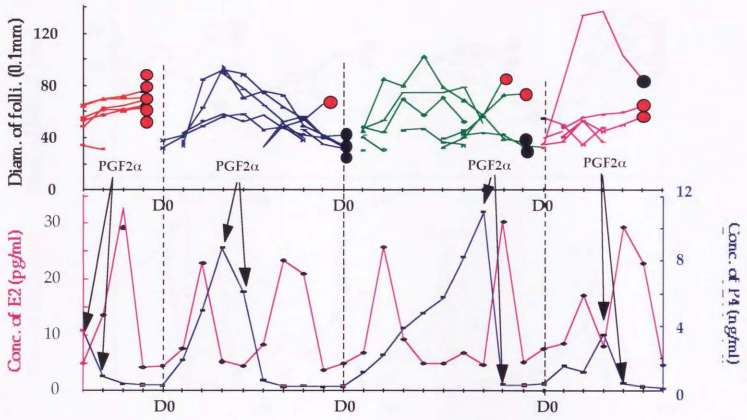


Fig.3.4 Induction of incomplete estrous cycles in a Shiba-goats #4. Changes in follicular diameter (upper panel) and serum progesterone and estradiol concentrations (lower panel). D0: Day of ovulation

● Ovulated follicles ● Atretic follicles

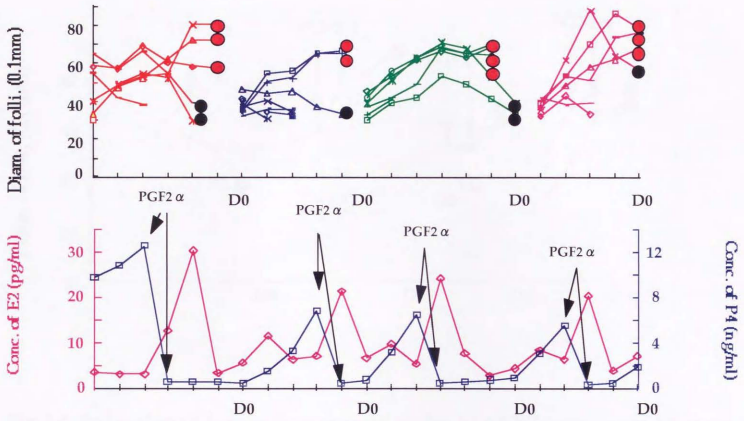


Fig.3.5 Induction of incomplete estrous cycles in a Shiba-goats #5. Changes in follicular diameter (upper panel) and serum progesterone and estradiol concentrations (lower panel). D0: Day of ovulation

● Ovulated follicles ● Atretic follicles

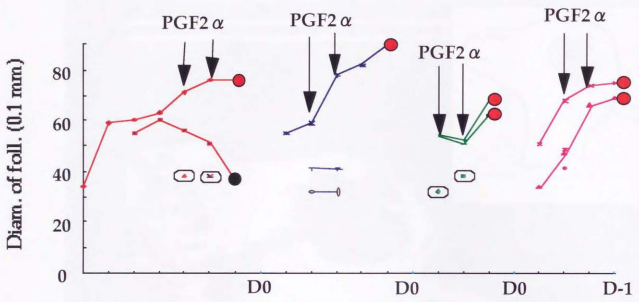


Fig.3.6 Induction of incomplete estrous cycles in a Shiba-goats #6. Changes in follicular diameter. D0: Day of ovulation

● Ovulated follicles ● Atretic follicles

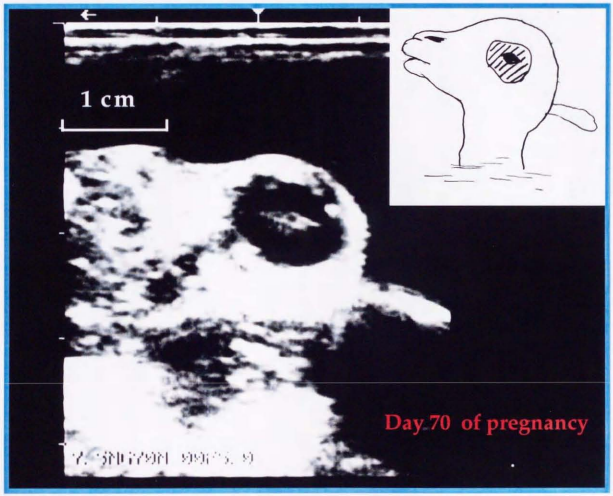


Fig. 3.7 Ultrasonographic observation of the fetus

General Discussion

General Discussion

In Chapter 1, changes in body weight and peripheral steroid hormone levels were monitored in both sexes from birth to 50 weeks of age. The first ovulation in females occurred earlier in animals with larger body weight and 3 of 5 females with smaller body weight in this study did not ovulate until 50 weeks of age. The age of the first mating was decided by detection of the first evident estrous behavior, and was also well correlated with body weight at 50 weeks of age. Arije and Wiltbank (1971) reported that body weight among heifers at puberty was considerably standardized, because animals with smaller body weight reached puberty at older age. Although this was also the case in this study, body weight at the first mating was still much variable. Body weight of the first mating in smaller goats was much smaller than larger goats, indicating that the body weight is not the direct determinant of onset of puberty, although it has substantial influence.

Serum estradiol concentrations were extraordinary high at the day of birth then declined to variable levels between 2 to 10 pg /ml from 2 to 50 weeks of age. The exact reason for high estrogen levels in neonatal goats will be an subject of the future study. Variation in estradiol levels during prepubertal period was also observed in goats by Venturina and Obsioma (1994) and in heifers by Adams *et al.*, (1994) who suggested that follicles would start to grow and this growing wave would recur during prepubertal period. Two female goats at 4 and 16 weeks of age in this study were found

to have middle to large sized follicles in their ovary by ultrasonography. Thus in goats, follicular development would occur irregularly from very early age. Irregular elevations of estradiol levels in each animal would indicate irregular follicular growth which did not result in ovulation.

This follicular growth should be induced by gonadotropin stimulation. If so, the hypothalamo-pituitary-gonadal axis in goats seems to be active form very early age, because sporadic elevations of estradiol concentrations were observed from 4 weeks of age. This feature is in contrast with human or primate species where gonadal activity is completely suppressed for a certain period of time before puberty. Some essential mechanisms of the onset of puberty might be different between ruminant and primate species, which will be an object of the future study.

Progesterone in female goats stayed continuously at precipitous levels before an abrupt increase which occurred later than 40 weeks of age. These elevated levels of progesterone concentration can be attributable to the ovulation and following formation of the functional corpus luteum.

It is believed that progesterone increase is required beforehand for ruminant species to express complete estrous behavior (Gonzalez *et al.*, 1975). This pattern seems to be common to the resumption of estrous cycles after periods of anovulation, for example, in beef heifer at puberty, cows after parturition and sheep after seasonal anoestrus (Thorburn *et al.*, 1969). It can be said from this study that expression of estrous behavior is gradually intensified by post-pubertal young goats recurring a couple of ovulatory

cycles.

As a whole, testosterone levels in male goats gradually increased from birth to 50 weeks of age, associating with large fluctuations. These fluctuations may be resulted from once a week sampling which randomly hit a value between the peak and the nadir of pulsatile secretion of testosterone in these animals. Again as discussed in female goats for their changes in estradiol concentrations, the hypothalamo-pituitary-gonadal axis of male goats seems functional at their early life. The peak values exceeding 5 ng/ml appeared in 3 of the 5 males later than 34 weeks with larger body weight, but not in the rest of 2 goats with smaller body weight until 50 weeks of age. Endocrine maturity in male goats will be substantially influenced by their body weight as in female goats.

Presumable difference in some essential mechanisms of the onset of puberty between ruminant, and primate or rodent species made the author characterize the estrous cycle of the goat in the aspect of comparative endocrinology in the following two chapters.

In Chapter 2, dynamics of follicular development was studied by daily ultrasonographic examination during 2 successive estrous cycles. Then goats were treated with a luteolytic dose of prostaglandinF2α when the dominant follicle of first latent wave was expected to be in growing phase to see if this treatment successfully induced spontaneous ovulation.

Most of estrous cycles in Shiba goats could be characterized by growth

of one or two latent follicular waves during luteal phase, in concert with finding in cattle (Savio *et al.*, 1988; Knopf *et al.*, 1989; Adams *et al.*, 1992b; Sunderland *et al.*,1996). However, detailed observation of the estrous cycles with *one* latent follicular wave strongly suggested the potential presence of the second latent follicular wave. The second latent follicular wave in this category is characterized by insufficient growth of follicles. The maximum diameter stayed less than 4 mm, while that in the "two-latent-waves" exceeded 6 mm. Similar length of the estrous cycle between "two-latent-waves (20.8±1.7 days)" and "one-latent-wave (20.3±1.0 days)" also suggests that the basic composition of these two cycles are the same.

Using goats with two latent follicular waves, plasma progesterone and estradiol levels were correlated with the follicular growth dynamics. Plasma progesterone levels began to increase one day after ovulation (Day 1), attained at maximum plateau levels from Day 4 to 12, and then started to decrease. The source of these elevated levels of progesterone is derived from the corpus luteum which has formed after ovulation in the dominant follicle(s) of the third follicular wave of the antecedent estrous cycle. During the luteal phase when high progesterone levels were maintained, follicular growth seemed to be suppressed in general, but alternation of the cohorts of follicles occurred certainly as mentioned above.

Most evident estradiol elevation was observed immediately before ovulation of the dominant follicles derived from the 3rd follicular wave. This largest elevation of estradiol level will be responsible for induction of

ovulatory gonadotropin surge by stimulating GnRH surge generator in the hypothalamus. Much smaller elevations of plasma estradiol concentrations were observed when the dominant follicles derived from either the 1st or 2nd latent follicular wave reached at their maximum diameters. These small elevation of estradiol levels correlated well with the growth of follicles of each 1st and 2nd latent follicular wave.

It is well known that GnRH pulse generator activity is suppressed by progesterone to decrease its pulse frequency. Another aspect of regulation of gonadotropin secretion by the neural mechanism is that GnRH surge generator regulates the occurrence of ovulatory gonadotropin surge. GnRH surge generator is known to be stimulated by exposure to high levels of estradiol for a certain period of time and is inhibited by progesterone. Thus, the reason why the dominant follicles in the latent follicular wave does not ovulate is considered to be the presence of progesterone.

To confirm this hypothesis, goats were treated with PGF2α to induce luteolysis at the time when follicles in the first latent follicular wave grew enough to respond to enhanced gonadotropin secretion due to progesterone withdrawal. PGF2α injection induced an immediate depletion of plasma progesterone concentration, and an inverse elevation of plasma estradiol concentration, suggesting an increment of frequency of GnRH pulse generator. As a consequence, all of the 6 goats thus treated ovulated 2 or 3 days after PGF2α injection.

If the ovulation-inducing effect of the $PGF_{2\alpha}$ treatment is solely

attributable to a depletion of peripheral progesterone levels due to its luteolytic action, simultaneous progesterone treatment with $PGF_{2\alpha}$ should nullify the ovulation-inducing action of $PGF_{2\alpha}$. In all the 6 goats treated for two successive days with progesterone (40 and 20 mg) simultaneously with the $PGF_{2\alpha}$, induction of ovulation was blocked.

The results of Chapter 2 make the author to hypothesize that dominant follicles derived from all the follicular waves either latent or ovulatory can ovulate successively, if each precedent corpus luteum is obliterated by $PGF_{2\alpha}$.

The results obtained in Chapter 3 clearly demonstrated that each of the repeated inductions of luteolysis with $PGF_{2\alpha}$ allowed the formation of a follicular growth wave, immediately after the precipitation of peripheral progesterone levels. The dominant follicle(s) in this newly escalated first follicular wave could again ovulate spontaneously by induction of luteolysis in the preceding corpus luteum. After each induced ovulation, follicular growth started without any delay, and short estrous cycles recurred at 5-7 days intervals.

In a goat, 7 presumable follicular waves at 5 days' interval were recurred within 29 days of an experimental period. Although ovulation can be induced only 4 times, the frequency of these follicular waves suggests that goat should have an innate capacity to recur follicular growth at a short interval. Progesterone secretion from the preceding corpus luteum would

usually obscure this prototype feature of follicular growth dynamics in the goat.

Another rare example was observed in another goat. Because follicular growth after 2nd induced ovulation was so rapid, the 3rd $PGF_{2\alpha}$ treatment was omitted, and nonetheless ovulation occurred spontaneously 4 days later. Occurrence of this spontaneous ovulation can be ascribed to low plasma progesterone levels between the 2nd (induced) and the 3rd (spontaneous) ovulations. It may be still premature to narrate an exact reason for the absence of an elevation of progesterone level after the 2nd induce ovulation, but it seemed that the corpus luteum failed to acquire the function of progesterone secretion by some unknown reason.

Sawada *et al.*, (1994) reported that progesterone and 20α-OHP were detected in blood following ovulation in goats. The changing pattern of both steroids in goats are different from that in rats; the fluctuations in 20α-OHP after ovulation are roughly in parallel with those in progesterone, though 20 α-OHP levels are always lower than progesterone levels. Thus, expression of 20α–HSD activity in goats is considered to be ubiquitous or constitutive, but not specific to the stage of luteolysis as is so in rats. Because stage and tissue specific expression of 20α–HSD gene should be regulated by the regulatory sequence flanking to the gene, mutation in this regulatory portion in the goat and probably in cattle may change the dynamics of 20α-OHP secretion, the function of 20α–HSD gene, and consequently the phenotypes of the estrous cycle from the incomplete to the complete one.

The fertility of ovum and normal expression of estrous behavior was confirmed after repetitive induction of ovulations. These results suggest that the female goat is furnished with every necessary mechanism for recurring short estrous cycle (incomplete estrous cycle observed in rats and mice), if progesterone levels are kept nearly at nil.

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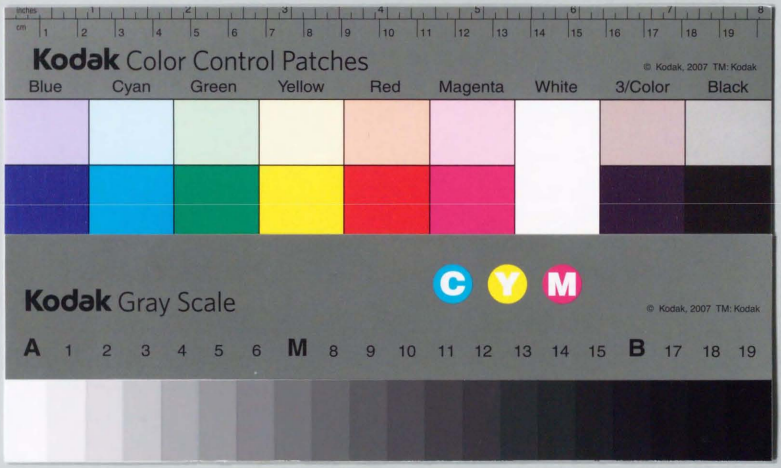
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