Studies on the Reproductive Functions in the Bull

（種雄牛の繁殖機能に関する研究）

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Contents

Chapter 1

General Introduction

1.1. Background

The global challenge of recent time is to increase the reproductive and production efficiency of food-producing animals, such as cattle, to meet the demand of increasing human population. Male animals share the equal genetic contribution to the offspring as females, and have become a major contributor of the genetic improvement. In cattle production, the utilization of proven tested sires through the recent advances of frozen semen and artificial insemination technologies has become the most convenient and successful tool for the genetic improvement of offspring in a wide range. This can be seen in the trend of improvement in milk production in relation with the artificial insemination (AI) coverage in Japan (Fig. 1.1). Early sexual maturation for early start of progeny testing, as well as obtaining a good number of quality spermatozoa are some of the most desirable reproductive functions of bulls. Many intrinsic factors such as the hormones of reproduction and the extrinsic factors such as the nutrition and thermal stress play the major roles on the development of sex organs including the testes and their functional status in bulls.

The early studies have explained about the developmental phenomena of sex organs (3, 48, 98, 163), hormonal regulation of reproduction (7, 9, 13, 41) and the effect of thermal stress (32, 94, 144, 161, 178) in the bovine testis. However, the postnatal structural regulations of seminiferous tubules and Sertoli cells, pubertal and postpubertal regulations of spermatogenesis, the effect of thermal stress on testicular development and functioning, and the sub-standard quality of spermatozoa are still the

major concerns in bull reproduction, which need further investigation. Moreover, the recent trend of research in the field of animal reproduction is focused more about females than males. It has been believed that the present study on reproductive functions in bulls and the data presented in this dissertation may contribute significantly in this endeavor.

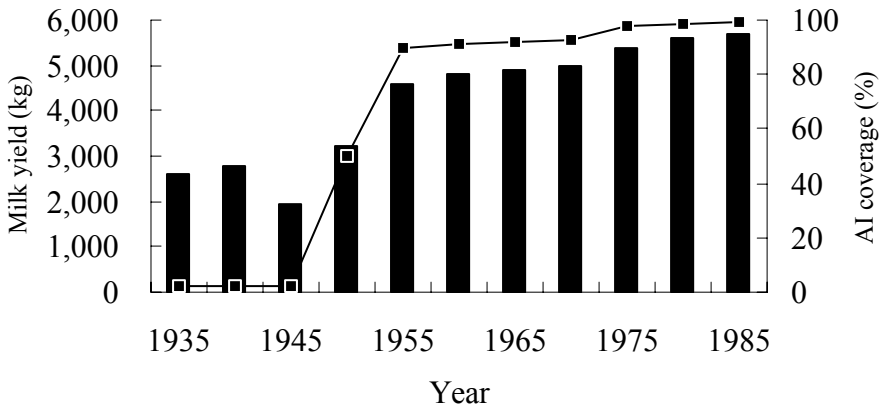


Fig. 1.1. Changes of milk yield (■) per year per cow accompanied with artificial insemination (AI) coverage (—■—) in Japan.

Source: Modified from Y. Nishikawa (1973). Artificial insemination in domestic animals (Jpn.). Vol. 35, 1~9.

1.2. Structure of the testis and regulation of reproductive functions in bulls

1.2.1. Testis structure

Testis is the main organ of male reproduction, which is responsible for the production of spermatozoa (spermatogenesis), sex hormones (steroidogenesis), paracrine/autocrine factors and fluid for spermatozoa transport. The morphological appearance of a mature bull testis is shown in Fig. 1.2A. On longitudinal incision, it reveals grossly a soft, tan mass of parenchyma and a central white longitudinal band of connective tissue known as the mediastinum (Fig. 1.2B). Histologically, parenchyma consists of avascular tubular compartment of seminiferous tubules and vascular interstitial compartment (Fig. 1.2C). The mediastinum houses the rete tubules or rete testis surrounded by the dense connective tissue (Fig. 1.2D). The terminal segment of seminiferous tubule, called the straight tubule, connects the seminiferous tubules with the rete tubules. The seminiferous tubules consist of the somatic supporting cells or Sertoli cells and various populations of germ cells such as the spermatogonia, which lie along the tubule wall, and the more mature series of spermatocytes, spermatids and spermatozoa, which lie successively towards the lumen. The interstitium consists of the Leydig cells, connective tissue, blood vessels and lymphatics. The contractile myoid peritubular cells surround the seminiferous tubules. Seminiferous tubules are the production house of spermatozoa that are ultimately transported out of the testis into the epididymus through rete testis and efferent ducts. The testis is a classic evolution in which spermatogenesis and steroidogenesis are sustained by a multitude of interactions between each cell and an elaborate intercellular communication system coordinates efficiently the different cell types to ensure orderly spermatogenesis. Sertoli cells, which undergo morphological differentiation from immature type into the mature type during

first 28 weeks of proliferative development, have a broad spectrum of functions in the physiology of spermatogenesis (163).

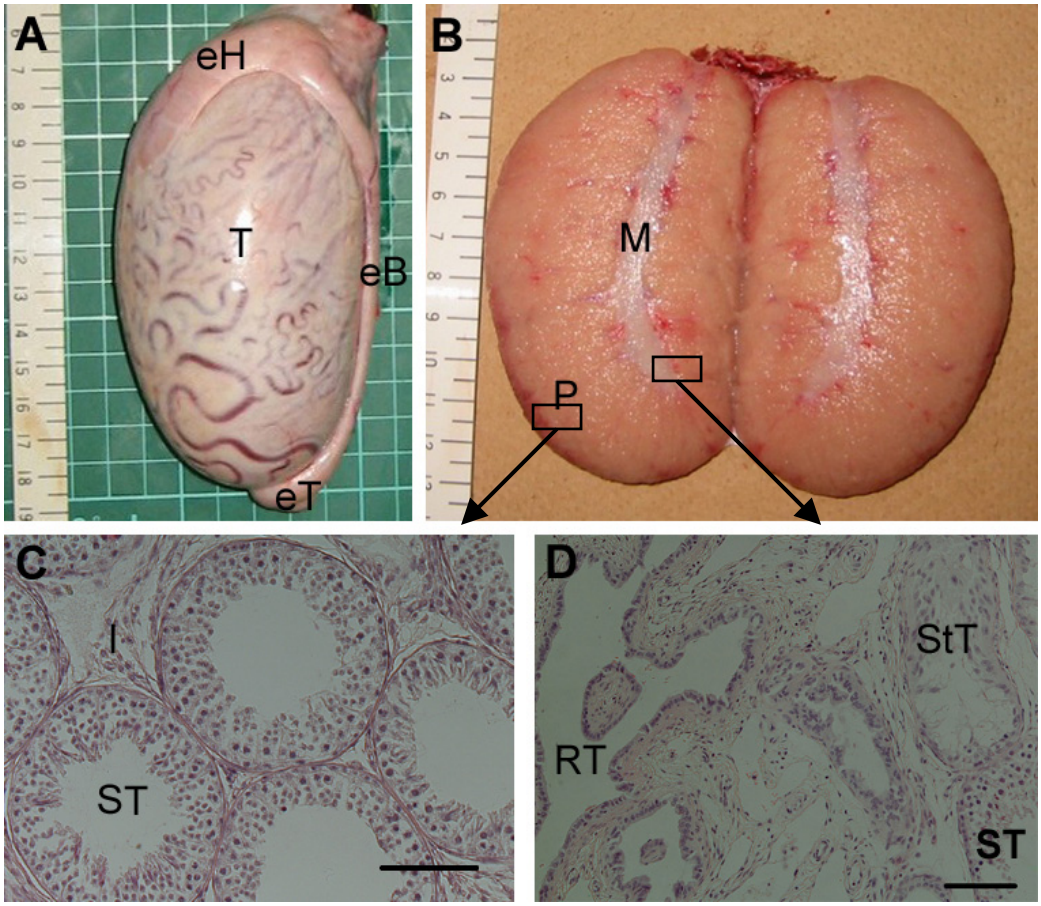


Fig. 1.2. A. Testis (T) of a 14-month-old Holstein Frisian bull with the epididymus intact (eH: head, eB, body and eT: tail of the epididymus). B. Longitudinal section of the testis (P: parenchyma and M: mediastinum). C. Hematoxylin-eosin (HE) stained histological section of the testis prepared from the parenchyma (ST: seminiferous tubule, I: interstitial cells). D. HE stained histological section prepared from the junction of parenchyma and mediastinum (ST: seminiferous tubule, StT: straight tubule or terminal section of seminiferous tubule, RT: rete testis). Bars: 100 μm.

1.2.2. Endocrine regulation of the male reproduction

Reproduction is regulated by a remarkable interplay between the nervous system and endocrine system. The fundamental responsibility of the nervous system is to translate external stimuli into neural signals that bring about a change in the reproductive organs and tissues (159). Endocrine regulations of testicular functions involve the changing patterns of secretion of the hypothalamic hormone called gonadotrophin-releasing hormone (GnRH), the anterior pituitary gonadotrophins called luteinizing hormone (LH) and follicle stimulating hormone (FSH), and the testicular steroids called androgens and estrogens. Endocrine regulation is important to ensure that the reproductive processes are synchronized with physiological events.

The endocrine regulation of reproduction in male animals is shown in Fig. 1.3. GnRH regulates the production of LH and FSH, which in turn regulate the production of gonadal steroids. LH binds to the receptors in the Leydig cells and FSH binds to the Sertoli cell receptors. Leydig cells produce testosterone that is transported to the adjacent vasculature and the Sertoli cell where testosterone is converted to dihydrotestosterone. Moreover, testosterone in the Leydig cells or in the Sertoli cells is also converted to estradiol. Testosterone and estradiol are transported by the blood to the hypothalamus where they exert a negative feedback on the GnRH neurons. Sertoli cells also produce inhibin, which exert negative feedback on FSH production by the anterior pituitary gland.

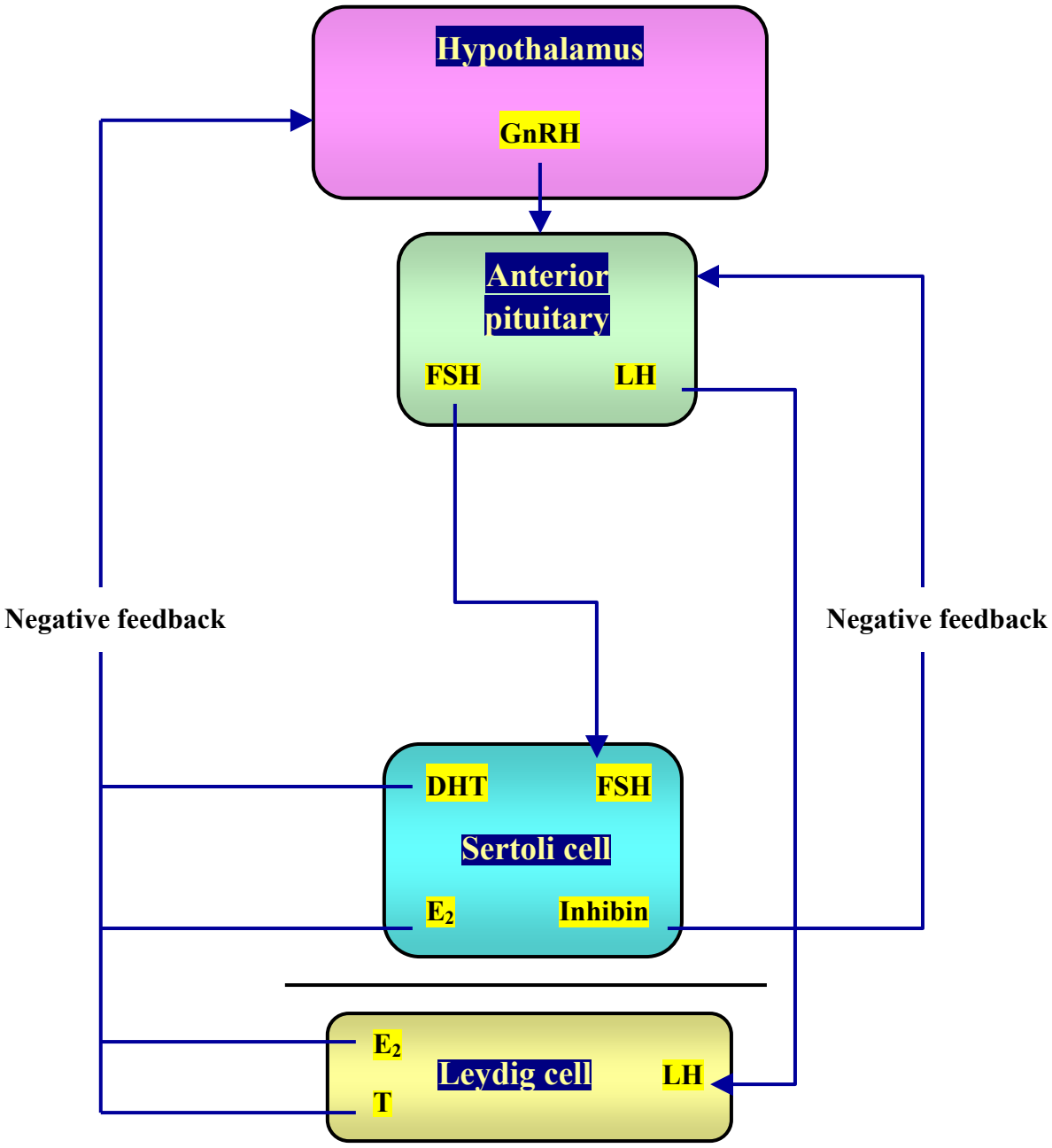


Fig. 1.3. Endocrine regulation of the reproductive functions in male animals. DHT: dihydrotestosterone, E_2: estradiol, FSH: follicle stimulating hormone, LH: luteinizing hormone, T: testosterone.

1.2.3. Endocrine regulation of the postnatal testicular functions

The hormonal and cellular events during postnatal testicular development, onset of spermatogenesis, puberty and postpubertal maturation has been well established in Holstein bulls (3, 7, 9, 13, 41, 48, 98, 156), which is summarized and shown in Fig. 1.4 and Fig. 1.5. The most precise explanation from birth through prepubertal period of sexual development (9) includes an early transient rise of LH at around 12 weeks of age that induces the differentiation of Leydig cells resulting an initial increase of androgens, androstenedione followed by testosterone. Testosterone subsequently induces the morphological differentiation of Sertoli cells concomitantly with the differentiation of gonocytes to pre- and A-spermatogonia. These events follow the increased sensitivity of the hypothalamus-anterior pituitary to negative feedback of gonadal steroids that causes the diminished frequency and amplitude of LH discharges. The cessation of Sertoli cells formation followed by formation of junctional complexes between adjacent Sertoli cells and establishment of the blood testis barrier is then subsequently followed by the formation of primary spermatocytes and ultimately spermatids and spermatozoa. The efficiency of spermatogenesis keeps increasing until attainment of sexual maturity.

Androgens are essential for virilization of the male urogenital tract during embryogenesis and masculinization towards the male phenotype at puberty (33, 99). However, the role of estrogens in bull reproduction is not clearly understood.

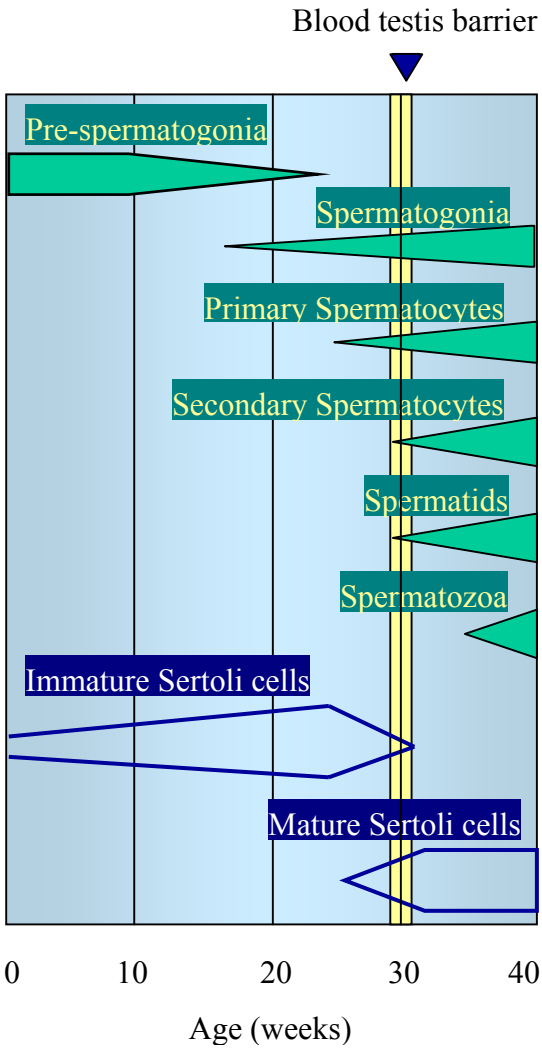


Fig. 1.4. Postnatal series of cellular development in the bovine testis

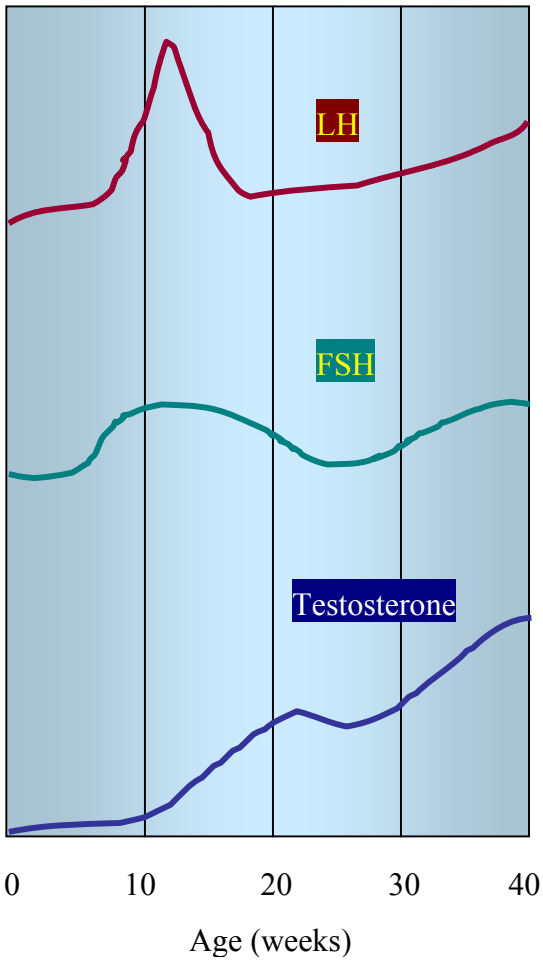


Fig. 1.5. Postnatal changes of hormonal concentrations in bulls

1.2.4. Scrotal size, semen quality and endocrine regulation around and after puberty

Achieving early puberty and obtaining good quality spermatozoa postpuberty have tremendous merits in bulls. Spermatozoa are collected from postpubertal Holstein bulls between 10 to 12 months of age, which allows to evaluate their early reproductive function to produce good quality spermatozoa sufficient to participate in the progeny-testing program as early as possible. Early collections from young bulls are often substandard in terms of sperm concentration, motility, or morphology (47, 57, 98). However, some improvement in quality and quantity of spermatozoa occurs with advancing age (6). Of the traits that are assessed during physical examination, those most closely correlated with fertility are scrotal circumference (SC) and the semen quality parameters of morphology, concentration and motility percentage of spermatozoa (40, 45, 70, 132). Similarly, measuring SC is particularly important in the examination of yearling bulls and it is highly correlated with sperm production and semen quality (30). It has been established that the pubertal index of SC in bulls regardless of the breeds is 27.9 ± 0.2 cm (105).

Gonadotrophins and gonadal steroids together regulate spermatogenesis and various other reproductive functions. It is postulated that a threshold level of testosterone is required for a bull to display mating behavior (28). Low testosterone level can maintain quantitatively normal spermatogenesis (103, 160). Positive relation (133)), or no relation (62, 134) has been shown between testosterone and semen production in bulls.

Estrogen is also produced by the testis. Different types of testicular cells in several species have been found to bear estrogen receptors. The emerging physiological

roles of estrogens in male fertility are reported in many recent studies. Recent publications (39, 78, 80, 82, 130) have highlighted the possible role of estrogens over androgens during spermatozoa maturation process in the efferent ductules and epididymus, which is important for sperm motility and fertilizing ability. Estradiol is also considered as germ cell survival factor in the human testis (137). It is, therefore, important to investigate the relationship among SC, semen parameters and endocrine parameters in the pubertal and postpubertal Holstein bulls to establish the regulating factors of spermatogenesis in yearling bulls.

1.2.5. Steroidogenic enzyme activity in the testis

Testis is the organ of steroid sex hormone production in the male. The biosynthesis of steroid hormones is shown in Fig. 1.6, which begins with the enzymatic cleavage of side chain of the substrate of cholesterol to form pregnenolone (135). This cleavage reaction is catalyzed by the cytochrome P450 side chain cleavage (P450scc) enzyme. Subsequently pregnenolone is metabolized by the enzymes 3β-hydroxysteroid dehydrogenase (3βHSD) and cytochrome P45017α-hydroxylase (P450c17). One of the Δ^4 or Δ^5 pathways can be followed from pregnenolone to androgens synthesis, and the preferred route may be both species- and age-dependent. In the final step, androstenedione is converted to testosterone. Estrogen biosynthesis, on the other hand, is catalyzed by cytochrome P450 aromatase (P450arom) enzyme, which converts androgen into estrogen. Therefore, the localization patterns of P450scc and P450arom enzymes may indicate the status of testosterone and estrogen productions respectively by the testicular cells, which is not yet understood for bulls under normal and various abnormal conditions.

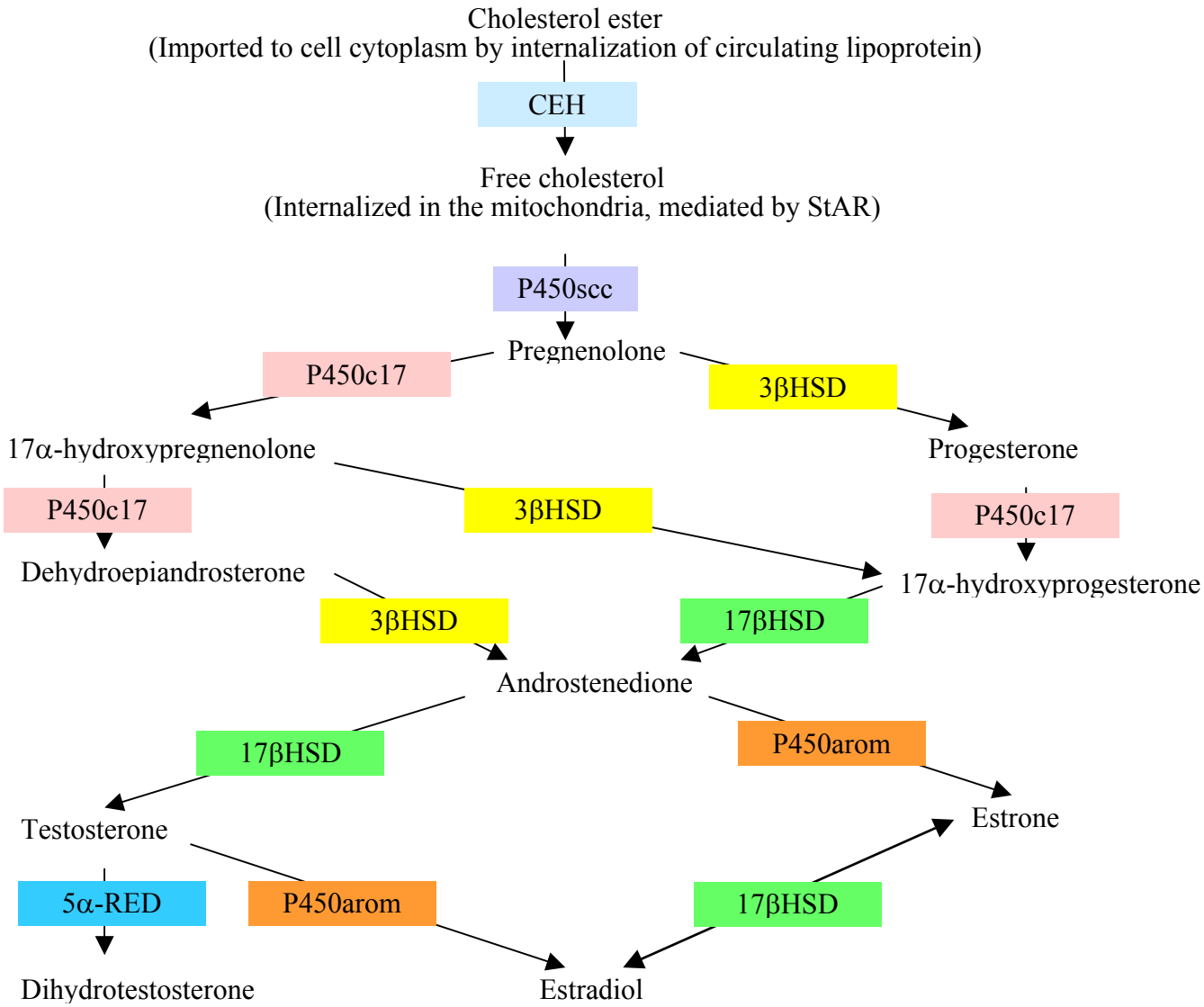


Fig. 1.6. Classical pathways of steroid biosynthesis.

CEH: Cholesterol ester dihydroxylase

StAR: Steroidogenic acute regulatory protein

P450scc: Cytochrome P450 side chain cleavage

P450c17: Cytochrome P450 17α-hydroxylase/C_{17-20} lyase

3βHSD: 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase

17βHSD: 17β-hydroxysteroid dehydrogenase (17-ketosteroid reductase)

P450arom: Cytochrome P450 aromatase, 5α-RED: 5α-reductase

1.2.6. Some cytoskeletal proteins and testicular structure and functions

The most important structural and cellular events leading to the initiation of postnatal spermatogenesis is the development and maturation of seminiferous tubules and the intratubular Sertoli cells. Cytoskeletal proteins provide structural and functional support to the cells by maintaining cell shape and intracellular environments (79). Some of the specific cytoskeletal proteins such as α-smooth muscle actin (SMA) and vimentin are localized in the seminiferous peritubular cells (131, 176) and Sertoli cells (63), respectively. These structural proteins may play the important roles on postnatal developmental regulations of seminiferous tubules and Sertoli cells. However, their precise role is not yet understood in the bovine testis. Mammalian Sertoli cells undergo morphological transformation during pubertal development (72, 163), which likely is regulated by vimentin in the rats (154, 187). Similarly, the expression of another cytoskeletal protein, known as cytokeratin, in the adult seminiferous epithelium has been regarded as a sign of either maintaining or regaining undifferentiated immature feature under various conditions such as the pathological testis causing fertility problems (15, 22, 114, 167, 168, 171), aging (52) in human, and cryptorchidism in human (153) and rhesus monkey (186). However, such relationships are not yet established in the bull. Therefore, the study of these cytoskeletal proteins may be useful to understand the changes in seminiferous tubules and Sertoli cells during postnatal development and different pathological conditions causing developmental alteration or bull fertility problems.

1.3. Testicular structure and functions under increased heat and pathological conditions

1.3.1. Bovine cryptorchidism

Cryptorchidism is a common congenital abnormality in which one or both testes fail to descend into the scrotum due to complex of genetic, hormonal, structural or other abnormalities. Descent of testis into the scrotum is possible only if the transabdominal descent followed by the inguinoscrotal descent is completed. Testis decent in many species such as human, pig, cattle and sheep is generally completed before birth, whereas in horse, rodents, lagomorphs, and dogs takes place postnatally (99). Fetal testis migrates from the posterior pole of the kidney and pass through the abdominal wall and descent into the scrotum (86). The series of events, which take place during testicular descent are: differentiation of fetal gonad into fetal testis that lies on the dorsal wall of the abdominal cavity and anchored to the ventral surface of the corresponding kidney by the cranial suspensory ligament, growth and transabdominal movement aided by the degeneration of the peritoneal fold that anchors the testis to the abdominal wall, regression of the Mullerian ducts elicited by Anti-Mullerian Hormone and the differentiation of the duct system induced by the steroid hormones secreted by the interstitial cells, movement of the gubernaculum, which is a peritoneum wrapped mesenchymal cord extending from the caudal end of the nephrogenic cord along the lateral abdominal wall to the developing scrotum, increase in abdominal pressure and herniation of the posterior peritoneal membrane to create an out-pocketing referred to as the vaginal process, which extends when the abdominal pressure increases, to form the inguinal canal along the course of the inguinal portion of the gubernaculum, dilatation of the inguinal ring, combined with the regression of the gubernacular ligaments and the

increasing intra-abdominal pressure forcing the flaccid fetal testis into the inguinal ring and finally the outgrowth of the gubernaculum through the inguinal canal guiding the movement of the testis and degeneration of the gubernaculum and contraction of the vaginal ring during the perinatal period causing the testis to descent into the scrotum.

Testicular descent is generally divided into two phases, an intra-abdominal and inguino-scrotal phase. The transabdominal descent is androgen independent and mediated by insulin-like peptide hormone INSL3 (5, 124, 188), whereas the inguinoscrotal descent depends on androgen action (86). However, in the androgen-deprived male piglets (113) and in male rat pups (61), testes were retained in the abdominal cavity, which may be associated with epididymal malformation (113). Eventhough the earlier stages of gubernacular growth are not androgen dependent in some species, the final stage of testicular descent, accomplished by gubernacular regression, is androgen-dependant (104). Environmental factors such as prenatal exposure to estrogenic and anti-androgenic compounds could affect INSL3 gene or other gene and interfere gubernacular growth or its regression causing failure of transabdominal descent (125, 188). Cryptorchidism is relatively rare in bovine and the factors regulating testicular descent is not fully understood in this species (99). Cryptorchidism severely affects normal spermatogenesis due to the exposure of testes to body heat. Locally induced heat or cryptorchidism not only causes germ cell loss, but also impairs the structures and functions of the basement membrane, peritubular cells, and Sertoli cells (73, 74, 87, 97). Cryptorchid testis can also be a good model to study the effect of heat in the testis during postnatal development.

1.3.2. Scrotal insulation as a model of the induced thermal degeneration of testes

Testicular temperature in bull is maintained 4-5^{o}C below body temperature (95, 31). Scrotal insulation model is useful to induce increased testicular temperature, which results in decreased sperm production and semen quality parameters such as spermatozoa morphology, concentration and motility (32, 94, 178). Moreover, peripheral testosterone level after induced testicular degeneration by scrotal insulation in bulls (144, 161) and due to pathological testicular degeneration in rams (26, 27) was decreased. It was also reported that the increased testicular temperature reduces testosterone secretion by the rat testis (68). Similarly, thermal stress induced by *Trypanosoma congolense* infection and scrotal insulation in rams was reported with reduced testosterone production in response to hCG treatment (121). However, the reason of this reduced Leydig cell steroidogenesis by the testis under thermal stress is not clear and the possible effect on the enzymes involved in steroid biosynthesis need to be investigated.

1.3.3. Testicular pathology, semen quality and response to exogenous GnRH

Bull fertility is very important because one bull may serve around 20 females under natural service conditions or hundreds of thousands under artificial insemination program. Only a few bulls may be sterile, but many bulls produce less or substandard quality spermatozoa that may impair fertility. Testicular biopsies of infertile men are often characterized by a mixed atrophy, in which different types of spermatogenic lesions are found in adjacent tubules, ranging from full spermatogenesis up to ‘Sertoli cell only’ tubules within the same biopsy (22, 162). Some cases of mild to severe

spermatogenic lesions are also reported in bulls (69, 119). Different types of spermatogenic arrests leading to impaired fertility and their relationships with gonadotrophic and gonadal steroid hormones are described in men (18, 24, 110), rats (129) and bulls (29, 119).

Although moderately predictable for the absolute fertility level, semen quality parameters such as motility, sperm number and sperm morphology have been valuable in identifying bulls of very low fertility (180). Deficiency in gonadotropin and steroid secretion did not appear to be the cause of infertility in bulls, which points towards other abnormalities, including impaired gonadal responses to those hormones or secretion of intratesticular factors (119). The testosterone and LH response to exogenous GnRH had no relation with the semen production (2) and libido (35) of bulls. However, testosterone response to GnRH was considered more reliable predictor of bull fertility than other reproductive measurements such as semen quality and SC parameters (143). Moreover, the LH response to GnRH was a good predictor of fertility when combined with the indices of scrotal volume, libido score and body weight (139). Semen quality in aged Holstein bulls was enhanced by treating with $PGF_{2\alpha}$ and/or GnRH (56). The comparison of LH, testosterone and estrogen response to GnRH treatment between the good quality and poor quality spermatozoa producing Holstein bulls and the testicular pathology in relation with the hormonal response and semen quality improvement are not yet reported in bulls.

1.4. General objectives

1. Study of the normal postnatal developmental regulations of the seminiferous tubules and Sertoli cells based on the changes of the expression patterns of cytoskeletal proteins, such as α-SMA, vimentin and cytokeratin.
2. Study of the functional regulation of spermatogenesis around and after puberty.
3. Study of the developmental alterations of the seminiferous tubules and Sertoli cells due to increased heat and pathological lesions in the testis.
4. Study of the altered steroidogenesis in the testis caused by increased heat.
5. Study of the pituitary and testicular responses to exogenous GnRH in bulls producing good quality spermatozoa and poor quality spermatozoa.

Chapter 2

Materials and Methods

2.1. Animals

The number of animals, samples used and animal's age in the present study are summarized in Table 2.1. Bulls reared in the Genetics Hokkaido (Shimizu, Hokkaido), National Livestock Breeding Center (NLBC, Nikkappu station, Shizunai), Obihiro University and various farms in Hokkaido, Japan were used.

Table 2.1. Number of animals, sample type and age ranges used in the present study.

Experiment	Number of animals, source	Sample used	Age range
1. Study of the normal conditions:			
Testis development	28, various	Testis	Birth to maturity
Regulation of spermatogenesis	12, NLBC	Blood, semen	Yearlings, around and after puberty
2. Study of the abnormal conditions:			
Cryptorchid condition	14, OU and various	Testis, blood	1-18 months
Scrotal insulation	2, NLBC	Testis, blood	12 and 14 months
GnRH treatment	9, GH and NLBC	Testis, blood, semen	1.5-7 years

GH: Genetics Hokkaido, NLBC: National Livestock Breeding Center, OU: Obihiro University

2.2. Estimation of semen quality and measurement of testis size

Semen was collected once or twice a week by using an artificial vagina and analyzed routinely to measure the volume, spermatozoa concentration, progressive motility after collection (MAC), and progressive motility after freezing and thawing (MAT) in Genetics Hokkaido and NLBC. These data were received from the farm and used to categorize the reproductive status of bulls based on their semen quality and used in the respective experiments.

The scrotal circumference (SC) of bulls used in the study of peripubertal and postpubertal regulation of spermatogenesis was measured using a retractable measuring tape as shown in Fig. 2.1.

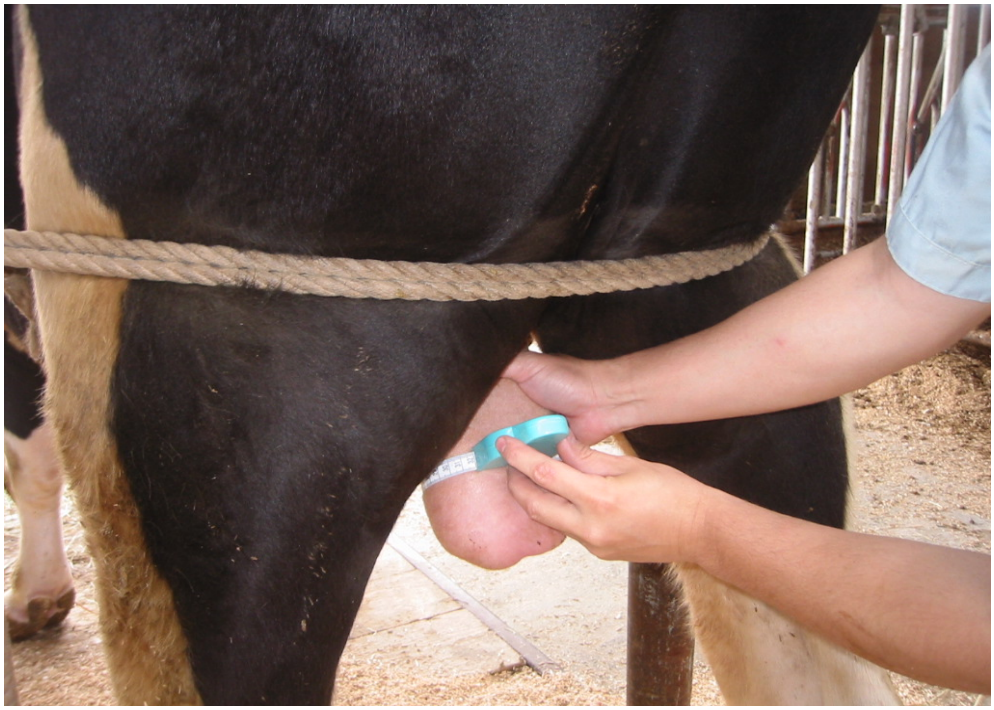


Fig. 2.1. Measurement of scrotal circumference in the bull.

2.3. Histological preparation

Testis samples were collected immediately after surgical castration or slaughter, dissected (1 cm x 1 cm) and fixed in Bouin's solution (saturated picric acid: 10%formalin: acetic acid = 15:5:1) for 24 hours and transferred to 70% alcohol for storage (Fig. 2.2). Sample blocks (1 cm x 0.5 cm) were prepared from the parenchyma and mediastinum and processed as shown in the following flow chart and in Fig. 2.2.

Dehydration into 70% to absolute alcohol (Ab) I - III, time: 12 hours each up to 95%, 4 hours into 99%, 2 hours into Ab I and 1 hour into Ab II and III

⬇

Clearance into Xylene (Xyl) I - III, time: 45 mins in Xyl I, 30 mins in Xyl II, III)

⬇

Paraffin embedding as Paraffin (Par) I - III, time: 30 mins each)

⬇

Blocking by mounting in paraffin and fixing in a wooden block

⬇

Sectioning (5 μm, by a gliding microtome)

⬇

Mounting (on gelatin or silicon coated glass slides).

Fig. 2.2. Dissection of the testis prior to fixing into Bouin's solution, paraffin blocking and sectioning with microtome.

2.4. Hematoxylin-eosin staining

The following routine procedure was applied to stain the sections by hematoxylin-eosin (HE) staining.

Deparafinization (Xyl I-III, Ab alcohol I to 70% alcohol, time 2 min each)

Washing (running water, RW: 10 min and distilled water, DW: 5 min)

Mayer's hematoxylin (30 sec to 1 min)

Washing (RW: 10 min)

4% eosin (30 min, in to shaker)

Dehydration (70% to Ab alcohol III, 1 min each), clearance (Xyl I - III, 2 min each), mounting (with DPX) and covering (with a glass cover slip)

2.5. Immunohistochemical staining

Immunohistochemical staining of the testis sections was carried out using the avidin-biotin peroxidase complex (ABC) method (84), or using Impress kit, as shown in the following flow chart.

Deparafinization (Xyl I-III, Ab alcohol I to 70% alcohol, time 3 min each)

Washing (RW: 10 min, DW: 5 min x 1 time)

Heat treatment:

Microwaving (500KW, for 20 min) by dipping sections into DW

OR

Water bath (98^{o}C, for 20 min) by dipping sections into retrieval solution, cooling into room temperature

Washing (RW: 10 min, DW: 5 min x 3 times)

Endogenous peroxidase blocking (1% of 30% H_2O_2 into methanol)

Washing (RW: 10 min, DW: 5 min x 3 times, PBS: 5 min x 3 times)

Section encircling (by water repellent PAP or DACO pen, DakoCytomation, Kyoto, Japan)

Normal goat (for polyclonal) or normal horse (for monoclonal) serum (1:50, 30 min at room temperature inside a humid box), or Impress reagent (30 min)

Normal serum drainage, primary antibody treatment (overnight, at 4^{o}C and inside a humid box)

Washing (PBS: 5 min x 3 times)

Biotinylated secondary antibody (1:200, anti-rabbit in goat for polyclonal antibody, or anti-mouse in horse for monoclonal antibody (BA-2000, Vector Laboratories, Inc., Burlingame, CA, USA), or secondary antibody from Impress kit, for 30 min at room temperature)

Washing (PBS: 5 min x 3 times)

Avidin-Biotin-Complex (1:2, PK-6100, Vectastain ®, *Elite* ABC kit, Vector Laboratories, Inc.)

Washing (PBS: 5 min x 3 times), for ABC method

0.2% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% H_2O_2 in Tris-HCl buffer (pH 7.4) prepared as 50 ml of Tris-buffer + 10 μl of 30% H_2O_2 +0.01 g DAB: mix for 20 min in a magnetic stirrer) treatment for ABC method, by controlling time

Washing (PBS: 5 min, DW: 5 min)

Counter staining (Mayer's hematoxylin, 1 or 2 dips)

Washing (RW: 10 min)

Dehydration, clearing and mounting

2.6. Blood sampling and hormone assay

Blood samples were collected in the heparinized vacuum tubes, centrifuged at 3000 rpm for 20 min to separate the plasma, which was aliquoted and stored at –30^{o}C until assayed.

The plasma LH concentrations were measured by a double-antibody radio-immunoassay (RIA) (93). The LH standard preparation and hormone for iodination was USDA- b LH-B-6 (USDA Animal Hormone Program, Beltsville, MD, USA). An antiserum to bLH was generated in guinea pigs (Laboratory of Animal Reproduction, Iwate University, Japan), and goat anti-guinea pig IgG serum (secondary antibody) was supplied by Dr. T. Matozaki (Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan).

Testosterone and E_2 assays were performed after diethyl ether extraction of steroids, which were reconstituted in neutral buffer as undiluted, or 10 times diluted for testosterone, and 20 times concentrated for E_2. Samples were analyzed in duplicate using a second antibody enzyme-immunoassay (EIA) as described elsewhere (4). The average recovery rate of testosterone and E_2 were 76% and 85% respectively. The standard curve in testosterone-EIA ranged from 0.025 to 25 ng/ml, and for $E_{2,}$ ranged from 1.95 to 2000 pg/ml.

2.7. Preparation for cytogenetic studies

Peripheral blood samples at 30 weeks of age from cryptorchid bulls (n=2) were used for routine cytogenetic analysis. One ml of blood was incubated with 3 ml complete lymphocyte culture medium (10% fetal bovine serum in MEM; Invitrogen/GIBCO, and PHA) at 37^{o}C for 72 hours. Metaphases were harvested by

adding 20 µl of colcemid for 90 minutes, followed by hypotonic (0.5%, 0.075 M) KCl treatment for 20 minutes. It was then centrifuged in 1200 rpm for 5 min, decanted and repeated the treatment. Fixation was done using standard 3:1 methanol-acetic acid fixative (WAKO Pure Chemicals). Supernatant was drained out and sediment mixed with a little volume of fixative was dropped in a glass slide, air-dried and stained by 2% Giemsa stain for 20 minutes. Cytogenetic analysis was done under high-resolution oil immersion lens (Nikon Corporation, Japan).

2.8. Statistical analysis

The results of histological and immunohistological stainings were examined under light microscope (MICROPHOT-FX, Nikon, Japan) fitted with a digital camera and a display monitor. The expression patterns after immunohistochemical staining were tabulated and analyzed as positive (+) and negative (-), and the intensities as +, ++ and +++.

JMP 5.0.1 software (SAS Japan) was used for statistical analysis and the probability values $P<0.05$ were considered as significant. Data of scrotal measurements, semen parameters and hormone concentrations were pooled and analyzed by ANOVA. Repeated measure analysis was applied to analyze the time-group effects and their interactions on hormone levels during regular interval sampling period. Student's t-test was applied, in general, to compare the means between two groups. The pair-wise correlations by group were determined using multivariate analysis while establishing the relationships among various parameters in pubertal and post pubertal bulls.

Chapter 3

Normal Testis: Histology and Immunohistochemical Expression Patterns of α-Smooth Muscle Actin (SMA) and Vimentin During Postnatal Development

3.1. Introduction

During postnatal sexual development of bulls, first 10-12 weeks is the period of infancy. The rapid increase in testis size thereafter is due to the internal cellular changes brought about by the changes in endocrine axis. Rapid increase in the seminiferous tubule diameter and differentiation of pre-spermatogonia into A-spermatogonia around 16-weeks, appearance of the lumen around 20 weeks, and progressive proliferation of spermatogenic cells under the influence of testosterone after 16 weeks of age giving rise to primary spermatocytes around 24 weeks and spermatozoa at around 32 weeks of age are the major postnatal developmental and cellular events in the bovine seminiferous tubule (7, 9, 48, 58, 163). Similarly, Sertoli cells that have a broad spectrum of functions in the physiology of spermatogenesis, undergo morphological transformation from indifferent or presumptive type to mature Sertoli cells between 20-28 weeks of age, which includes distinct changes in cell shape, nucleus and cellular organelles (163). Moreover during transformation, nuclear volume of the Sertoli cells increases and the irregular nuclear outline is achieved (185). In bulls, generally the immature indifferent Sertoli cell nuclei are round, located basally and termed as basal indifferent supporting cells. At the beginning of cellular differentiation some nuclei assume a pseudostratified disposition towards the center and termed as central indifferent supporting cells (3, 48).

The cytoskeleton is an active cytosolic protein framework comprised of

microfilaments (actins; diameter: 6-7 nm), intermediate filaments (cytokeratins, desmin, and vimentin; diameter: 8-10 nm), and microtubules (tubulins; diameter: 25 nm). Their roles in cellular structure and function include maintaining cell shape and polarity, positioning of intracellular organelles, forming of cytoplasmic extensions, and anchoring of organelles to the plasma membrane (79). Actin microfilament is one of the most conserved eukaryotic proteins and is expressed in mammals and birds as six isoforms: the α–skeletal muscle actin, the α–cardiac muscle actin, the α– and γ–smooth muscle actins, the α–and β–cytoplasmic actins and the two non-muscle actins (164, 118). Vimentin is found in most of the differentiating and fully differentiated cells. Generally actin filaments are the most flexible filaments, which have an essential role on structural and motility related functions whereas due to insolubility of vimentin, it is suggested that it serves a structural function in the cytoplasm.

As a specific marker of smooth muscle differentiation (164), seminiferous peritubular α–smooth muscle actin (SMA) has been detected in the first few postnatal days in the rat testis (131, 176) and in the early postnatal ovine testis (170). Increasing patterns of α–SMA in the ovine testis (170) and changes in the actin filament arrangement in the rat testis (108) during postnatal development have suggested a relationship for SMA with testicular development and functions. However, no postnatal localization patterns and possible functional relationships for SMA in the bovine testis have been reported so far.

Sertoli cells posses a highly organized cytoskeleton, and vimentin, the most common intermediate filament (63, 177), is located around the nucleus and provides it with structural support (15, 169, 170, 187). Sertoli cells transformation is likely related to cytoskeletal changes (154, 187). Increases in Sertoli cell vimentin in the prepubertal

ovine testis (170) and changes of its distribution area during postnatal development in rats (187) have indicated vimentin activity during postnatal development. Although perinuclear localization of vimentin has been demonstrated in the pre-Sertoli and adult Sertoli cells of the bovine testis (169, 184), its distribution and role during pubertal development are not yet understood. The objectives of the present study were to investigate the postnatal developmental changes in localization of α-SMA and vimentin in the bovine testis and to discuss their functional relationships.

3.2. Materials and Methods

The present investigation was carried out on 58 testes (0 months to 7 years old) collected from Holstein bulls (n=36) reared in Hokkaido and Iwate prefectures in Japan. The normally growing scrotal testes (n=8) from unilateral cryptorchid bulls were also included in the study. The collection of testis samples, their histological preparations and staining with hematoxylin-eosin (HE) stain and immunohistochemical technique with α-SMA and vimentin was performed as explained in Chapter 2.3, 2.4 and 2.5. Mouse monoclonal anti-α-smooth muscle actin (1:5000; Clone 1A4, Sigma Chemical Co., St. Louis, USA) and polyclonal anti-calf lens vimentin (1:150; MEDAC, Hamburg, Germany) were used as primary antibodies. The biotinylated secondary antibodies used were the mouse IgG raised in horse (1:200) and the rabbit IgG raised in goat (1:200) (BA-2000, Vector Laboratories, Inc., Burlingame, CA, USA). The control sections were treated with normal horse and normal goat serum instead of α-SMA and vimentin primary antisera, respectively. Similar procedures were followed for all samples.

3.3. Results

3.3.1. Histological changes in the postnatally developing seminiferous tubules

No major cellular changes in the seminiferous tubules were observed until 3 months of age and the spermatogenic cells observed were immature gonocytes (Figs. 3.1A, B). Until 4 months of age, all Sertoli cell nuclei were basal and indifferent, however, few dividing spermatogonia were observed at 4 months (Fig. 3.1C). Pre-spermatogonia and A-spermatogonia constituted the spermatogenic cells at 5 months and some Sertoli cell nuclei were displaced towards the lumen, referred as the central indifferent Sertoli cells, indicating Sertoli cells starting morphological transformation (Fig. 3.1D). It was followed by the progressive division of spermatogenic cells and transformation and maturation of Sertoli cells with nuclei rearranging basally around 8 months of age (Fig. 3.1E), and then onwards with full spermatogenesis supported by the mature Sertoli cells (Figs. 3.1F, H, I). Delayed maturation during postnatal series of cellular progression can easily be identified on the basis of position and shape of Sertoli cell nuclei and observation of the most advance stage of spermatogenic cells (Fig. 3.1G).

3.3.2. Postnatal immunohistochemical expression patterns of α-SMA and vimentin

Summaries of the immunohistochemical localization patterns of α-SMA and vimentin in the bovine testes during postnatal development are shown in Tables 3.1 and 3.2. The spermatogenic cells, Sertoli cells, and Leydig cells showed negative immunoreactions to α-SMA at all ages. Actin filaments of the walls of blood vessels were always positive (Fig. 3.2A-H), and were considered as the positive control. Bovine

testicular cells that showed developmental changes in their α-SMA immunoreactivities were the seminiferous peritubular myoid cells, sub-epithelial myoid cells of the straight tubules and rete testis, and stromal myoid cells in the rete testis. The immunoreactivities of the peritubular cells were negative from birth (Fig. 3.2A) until 3 months of age (Fig. 3.2B). Similar pattern was observed in the straight tubules and rete testis during this period (Fig. 3.2C). In the peritubular cells of 4-month-old testes, however, an intermittent positive reaction was observed (Fig. 3.2D). At this age, immunoreactions were weak in sub-epithelia and very weak in stroma of the straight and rete tubules (Fig. 3.2E). This indicates that α-SMA appears around 4 months of age in the bovine testis. All testes from 5 months old (Fig. 3.2F) or older bulls (Fig. 3.2G) demonstrated regular positive α-SMA around the seminiferous tubules, termed as the mature pattern. However in the straight tubules and rete-testis, the intensity increased gradually until 12 months of age and assumed the mature pattern (Table 3.1, Fig. 3.2H).

With regard to vimentin immunoreactions, spermatogenic cells were negative and a few interstitial cells, peritubular myofibrils, and blood vessels were positive with an unchanged pattern throughout postnatal development (Fig. 3.3A-G). Similarly, the positive immunostaining pattern in the basal part of the epithelia of the straight tubules and rete testis also did not change (Fig. 3.3H, I). Furthermore, bovine Sertoli cells were the only cells to show remarkable changing patterns during postnatal development. Testes from 0-day-old bulls (at birth) demonstrated weak positive immunoreactivities in the narrow basal perinuclear zone of the Sertoli cells (Fig. 3.3A) that increased moderately until 4 months of age (Fig. 3.3B). A remarkable increase in immunostaining intensity and changes in the pattern was observed in 5- to 8-month-old testes. This was characterized by bundles of prominently stained vimentin extending from Sertoli cell

perinuclei to the basal membrane (Fig. 3.3C, D). During this period, Sertoli cells were undergoing a transformation process, and their nuclei were at different distances from the basal membrane. Infranuclear vimentin filaments were observed supporting the Sertoli cell nuclei and connecting them to the basal membrane. A somewhat similar pattern was also observed in the testes of one 10-month-old bull that showed delayed maturity (Fig. 3.3F). Shortening of vimentin extension was noticed in 8-month and clearly observed in the 9-month-old testis (Fig. 3.3E) as the mature Sertoli cells appeared with nuclei positioned close to the basal membrane. This was followed by the appearance of the mature pattern of a strong and stable perinuclear Sertoli cell vimentin without any infranuclear extensions, but having a few nuclei bearing a short supranuclear flame (Fig. 3.3G).

3.4. Discussion

The histological observations in this study were generally consistent with previous reports for the bovine testis during postnatal development (7, 9, 48, 58, 163), except for one 10-month-old bull that showed delayed pubertal development. Application of microwave moist heat prior to immunohistochemical procedures probably enhanced the immunoreactive sensitivity in the present experiment. Although only qualitative, the present results clearly demonstrate the typical postnatal pattern of α-SMA and vimentin distribution in the bovine testis.

As shown in previous reports in the rat (131), monkey (157), human (14), domestic fowl (109), Japanese black bear (100), sheep (170), and bull (169) testis, results in this study demonstrated α-SMA localization in the seminiferous peritubular myoid cells and vascular smooth muscle cells. Moreover, the present results also

demonstrated α-SMA immunoreactions in the straight tubules and rete testis as well. The age of appearance of α-SMA in the bovine testis coincided with the age of initiation of postnatal division of spermatogonia, which starts around 4 months of age (48). This is in contrast with the postnatal detection of α-SMA in rats [131, 176] and sheep (170), which was observed during the first postnatal week and from early postnatal age, respectively. However, changes in the structural arrangement of peritubular actin filaments from circular to longitudinal have been reported in the rat testis in relation to the advancement of initial spermatogenesis (108). It has also been reported that structural actin (F-actin) in the bovine testis appears around 4 weeks of age in peritubular cells (184). It can be speculated that the α-SMA isomer appears relatively late. It has been suggested that actin filaments in peritubular myoid cells serve in a contractile capacity aiding the transport of spermatozoa in the tubular lumen (107). Appearance of α-SMA around 4 months of age in bovine peritubular myoid cells and immediate attainment of the mature pattern may help to increase contractility of seminiferous tubules and may also secrete some paracrine factors required for regulation of Sertoli cell functions (165) that are vital for spermatogenic process. Moreover, its relative delay in assuming the mature pattern in the rete testis may indicate that spermatozoa enter into the rete testis only during the later stages of puberty. It has previously been reported that appearance of α-SMA in the peritubular cells of monkey testes was induced by androgen (157). It can be speculated that androgen, which begins increasing around 4 months of age (7), may initiate the appearance of α-SMA and onset of postnatal division of spermatogonia in the bovine testis.

The present finding of Sertoli cell perinuclear localization of the vimentin is consistent with previous reports in the rhesus monkey (186), rat (15, 179, 187), human

(52, 111), South American camelids (152), sheep (170), and bull (184). The present observations of vimentin distribution from day-0 to 5 months of age and in mature bulls are in agreement with the previous studies in the bovine testis (169, 184). Similarly, as reported in rhesus monkeys (186) and in Japanese black bears (100), vimentin in this study was localized in a few interstitial cells and vascular walls of the bovine testes. Contrary to the observations in the Sertoli cells of the ground squirrel (10) and rat (187) and in agreement with previous reports in the ovine (170) and bovine (169), any changes in the distribution of vimentin during the seminiferous cycle were not observed. The most striking findings of the present study were the demonstration of a considerable increase in the vimentin immunostaining intensity and of changes in its pattern from 5 to 8 months of age when Sertoli cells were undergoing active morphological transformation (48, 163). The characteristic infranuclear vimentin bundles extending from Sertoli cell perinuclei to the basal membrane during this stage probably help in stabilizing the differentiating Sertoli cell nuclei, thus preventing dislocation of the nuclei and finally pulling them back close to the basal membrane around 9 month of age. This is in contrast with a previous study in the bovine testis (184) that reported negative vimentin immunoreactions at 30 and 40 weeks of age. This kind of discrepancy may be due to different embedding media being used in different experiments (184). Methodological protocols such as the application of microwave heat might have increased the sensitivity in this experiment. Although an increase in vimentin has been reported in the Sertoli cells of prepubertal sheep (170), the present results are more consistent with the findings for the postnatal day-14 rat, which had prominently stained vimentin bundles connecting the Sertoli cell nuclei to the basal membrane (187). However, the supranuclear vimentin, as observed in developing rats (187) were not

observed. Although a few mature Sertoli cell nuclei with a short supranuclear flame were demonstrated, as reported in mature sheep (170), they were not uniformly distributed and their role was not clear. The present results demonstrate a more specific role of vimentin in the positioning of bovine Sertoli cell nuclei during postnatal maturation. Furthermore, the vimentin localization pattern may indicate the maturation status of bovine Sertoli cells, as one 10-month-old bull showing relatively immature seminiferous tubules also demonstrated Sertoli cell infranuclear vimentin extensions similar to transforming Sertoli cells around 5 to 8 month of age. The characteristic vimentin localization pattern during pubertal development that was observed for the first time in this study might shed further light on the developmental study of postnatal bovine Sertoli cells.

Table 3.1. Summary of the immunohistochemical localization and intensity of α−smooth muscle actin (SMA) in bovine testes during postnatal development.

Age (month)	0	1	2	3	4	5	6	7	8	9	10	11	≧12
Number of testes	8	11	3	3	2	6		4	1	2	1		17
Number of animals	4	7	2	2	1	4		2	1	1	1		11
Spermatogenic cells	-	-	-	-	-	-	ND	-	-	-	-	ND	-
Sertoli cells	-	-	-	-	-	-	ND	-	-	-	-	ND	-
Interstitial cells	-	-	-	-	-	-	ND	-	-	-	-	ND	-
Peritubular myoid cells*	-	-	-	-	+	+++	ND	+++	+++	+++	+++	ND	+++
Sub-epithelial myoid cells of the straight tubules and rete testis	-	-	-	-	+	+	ND	++	++	++	++	ND	+++
Stromal myoid cells of the rete testis	-	-	-	-	±	±	ND	±	±	+	+	ND	+
In blood vessels	+++	+++	+++	+++	+++	+++	ND	+++	+++	+++	+++	ND	+++

-: Negative; ±: very weak, +: weak, ++: moderate and +++: intensely positive.

*: Intermittent at 4 months of age and regular at 5 months of age, and onward. ND: Not determined.

Table 3.2. Summary of immunohistochemical localization and intensity of vimentin in bovine testes during postnatal development

Age (month)	0	1	2	3	4	5	6	7	8	9	10	11	≧12
Number of testes	8	11	3	3	2	6		4	1	2	1		17
Number of animals	4	7	2	2	1	4		2	1	1	1		11
Spermatogenic cells	-	-	-	-	-	-	ND	-	-	-	-	ND	-
Sertoli cells (Perinuclear)*	+	+	+	++	++	+++	ND	+++	+++	+++	+++	ND	+++
Interstitial cells (a few)	+	+	+	+	+	+	ND	+	+	+	+	ND	+
Peritubular myofibrils	+	+	+	+	+	+	ND	+	+	+	+	ND	+
Basal part of the epithelia of the straight tubules and rete testis	++	++	++	++	++	++	ND	++	++	++	++	ND	++
In blood vessels	+	+	+	+	+	+	ND	+	+	+	+	ND	+

-: Negative; +: weak, ++: moderate and +++: intensely positive.

*: Characteristic infranuclear vimentin extensions from Sertoli cell perinuclei to basal membrane at 5, 7, and 8 months of age. ND: Not determined.

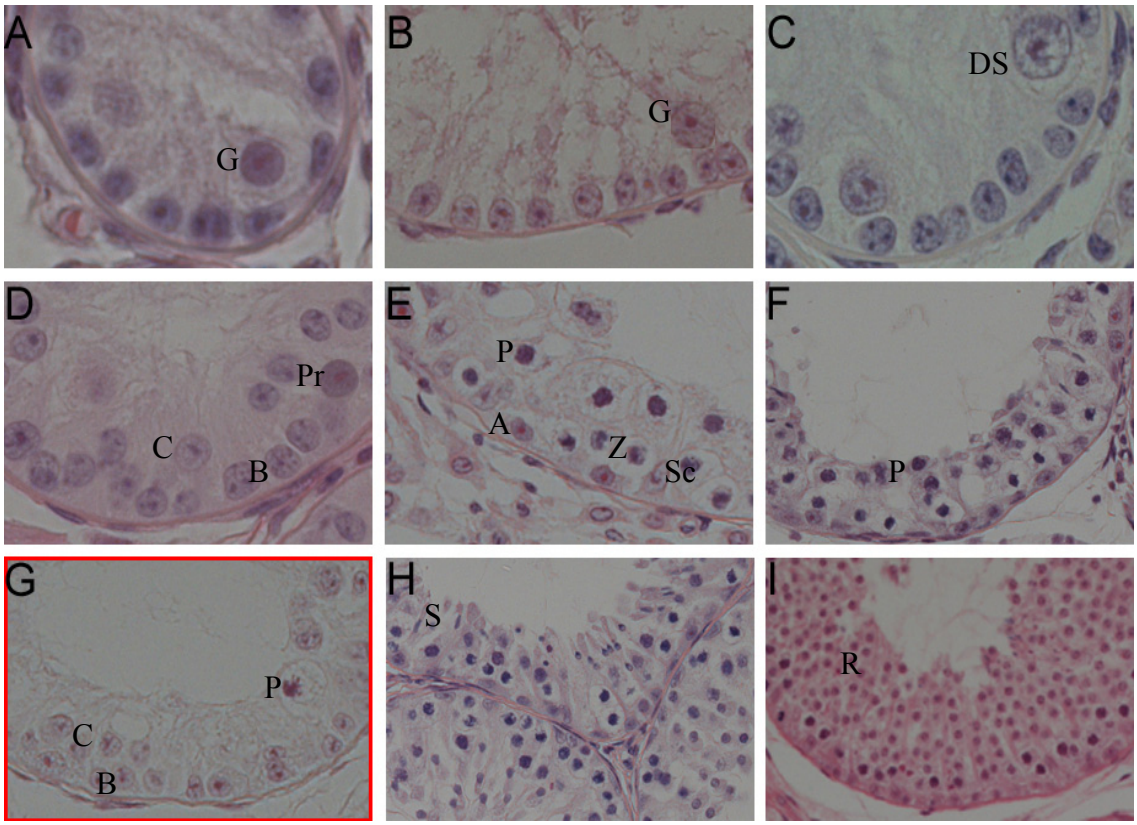


Fig. 3.1. Postnatal cellular changes in the seminiferous tubules of Holstein-Frisian bulls. Hematoxylin-eosin (HE) stained histological section of the testis from 1 month (A), 3 months (B), 4 months (C), 5 months (D), 8 months (E), 9 month (F), 10 months with delayed maturation (G), 12 months (H) and 18 months (I) bulls. A: A-spermatogonia, B: basal indifferent supporting cells, C: central indifferent supporting cells, DS: dividing spermatogonia, G: gonocytes, P: pachytene spermatocytes, Pr: pre-spermatogonia, R: round spermatids, S: elongated spermatid, Sc: Sertoli cell, Sp: spermatozoa, Z: zygotene spermatocytes.

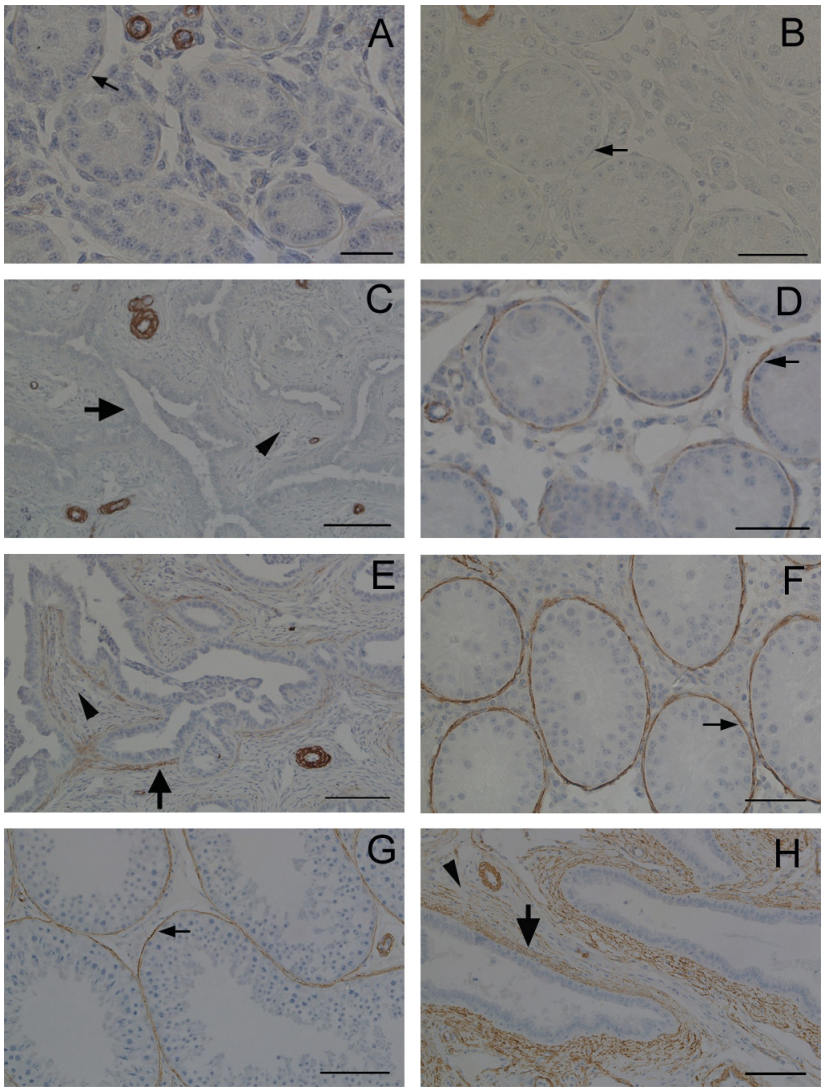


Fig. 3.2. α-SMA immunoreactions of representative bovine testes during postnatal development. The seminiferous peritubular myoid cells (small arrows) were negative on day-0 (A) and at 3 months of age (B), intermittently positive at 4 months of age (D), and regularly positive at 5 months of age (F) and 14-months of age (G). Sub-epithelial myoid cells of the straight tubules and rete testis (big arrows) were negative at 3 months of age (C), weak positive at 4 months of age (E) and intensely positive at 14 months of age (H), whereas stromal myoid cells (arrow heads) were very weak at 4 months of age (E) and weak at 14 months of age (H). Blood vessel walls were intensely positive at all ages (A-H). Bars: A: 25 μm; B, D, and F: 50 μm; C, E, G, and H: 100μm.

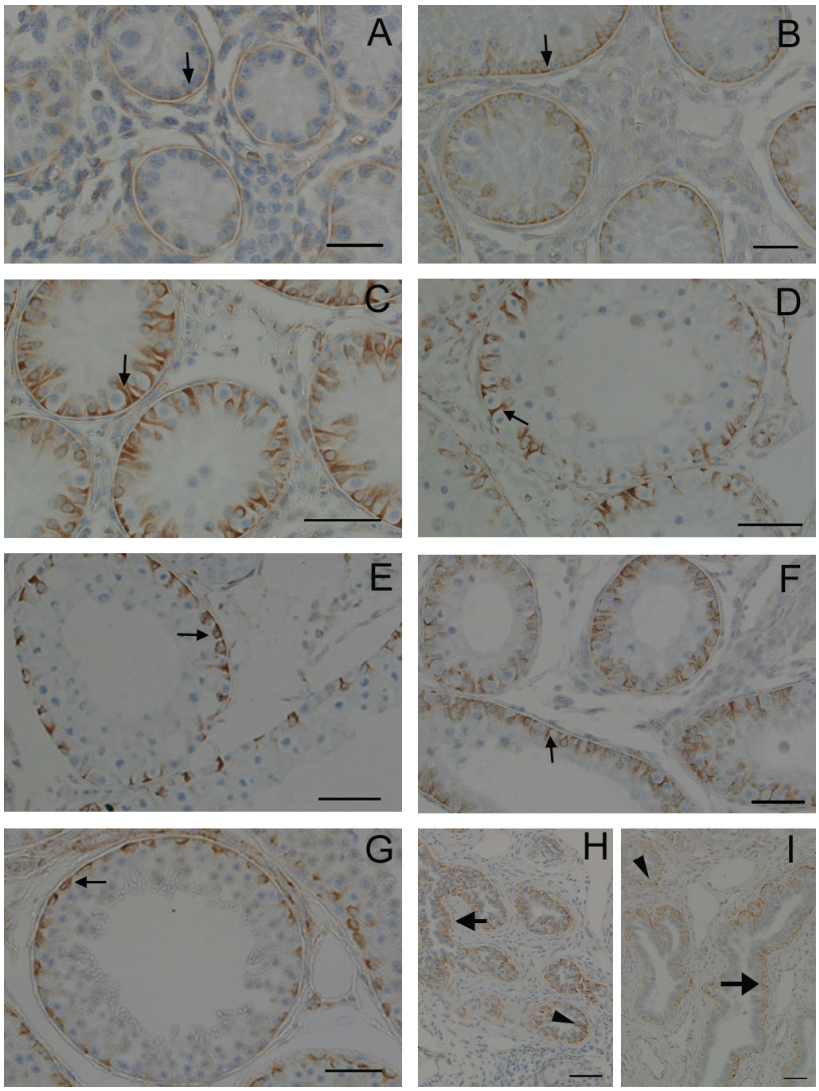


Fig. 3.3. Vimentin immunoreactions of representative bovine testes during postnatal development. The Sertoli cell perinuclear vimentin (small arrows) was basal and weak on day-0 (A), moderate in 4-months (B) and intense with infranuclear extensions connecting transforming Sertoli cell nuclei to the basal membrane from 5-months (C) until 8-months (D) and also in 10-month (F), which had immature seminiferous tubules as compared to 9-month-old testis (E). The infranuclear extensions shortened in 9- months (E) that almost resembled a 14-month-old testis (G). The positive patterns in the basal epithelia of the straight (arrow heads) and rete tubules (big arrows) were similar at 1 month (H) and 12 months of age (I). A few interstitial cells, blood vessels, and peritubular cells were weak positive at all ages (A-G). Bars: A, B: 25 μm; C-I: 50 μm.

Chapter 4

Functional Regulation of Spermatogenesis in Pubertal and Postpubertal Bulls

4.1. Introduction

Achieving early puberty and obtaining good quality spermatozoa postpuberty have tremendous merits in bulls. Spermatozoa are collected from Holstein bulls soon after puberty, between 10 to 12 months of age. It allows for evaluating their early reproductive function to produce good quality spermatozoa sufficient to participate in the progeny-testing program as early as possible. Early spermatozoa collections from young bulls are often substandard in terms of sperm concentration, motility, or morphology (47, 57, 98). However, some improvement in quality and quantity of spermatozoa occurs with advancing age (6, 140). Scrotal circumference (SC) and the semen quality parameters of morphology, concentration and motility percentage of spermatozoa are the most closely correlated traits with fertility of bulls (40, 45, 70, 132). Moreover, measuring SC is particularly important in the examination of yearling bulls and it is highly correlated with sperm production and semen quality (30). Bulls with SC measuring 27.9 ± 0.2 cm (105) or giving first ejaculate with 50 million spermatozoa having at least 10% progressive motility (98, 183) can be considered to have reached puberty.

Gonadotrophins and gonadal steroids together regulate spermatogenesis and various other reproductive functions. It has been suggested that a threshold level of testosterone is required for a bull to display mating behavior (28). Similarly, low levels of testosterone can quantitatively maintain normal spermatogenesis (103, 160). The

relationship between the circulating concentrations of testosterone and semen production in bulls is unclear, since positive relationship (133), or no relationship (62, 134) between them has been reported. Additionally, in several other species, some testicular cells have been found to bear estrogen receptors. The emerging physiological roles of estrogens in male fertility have been considered recently, particularly about the possible role of estrogens on the functional regulations of efferent ductules and epididymus (39, 78, 80, 82, 130), where spermatozoa mature and acquire motility and fertilizing ability. Estradiol is also considered as germ cell survival factor in the human testis (137). The aim of the present study was to investigate the relationships among the age, body weight, SC, semen parameters and the circulating levels of luteinizing hormone (LH), testosterone and estradiol-17β (E_2) in pubertal and postpubertal Holstein bulls, and to discuss the possible role of estrogen on spermatozoa motility.

4.2. Materials and Methods

4.2.1. Experimental design

Holstein bulls (n=12), aged from 8.5 to 18.0 (Mean ± SEM: 12.5 ± 0.8) months of age, reared and kept at National Livestock Breeding Center (NLBC), Niikappu station, Japan were randomly selected and subjected for weekly peripheral blood sampling for 6 weeks. Blood samples were collected in the same time every week to avoid pulsatile fluctuations of hormone concentrations, and plasma was harvested immediately at –30^{o}C until assayed for LH, testosterone and E_2. The SC was measured, once at the beginning of experiment and again after 4 weeks and the average SC was determined for each bull, which ranged from 22.5 to 34.2 cm (Mean ± SEM: 30.6 ± 1.2 cm). Monthly body weight data from the time of birth were obtained, which indicated

similar growth patterns in all bulls. Semen from these bulls, of the 6 weeks period of blood collection, collected once a week by using an artificial vagina was analyzed routinely at NLBC to measure the volume, spermatozoa concentration, progressive motility after collection (MAC), and progressive motility after freezing and thawing (MAT). The SC parameter of 27.9 ± 0.2 cm (105), followed by the first ejaculate quality of having 50 million spermatozoa with at least 10% progressive motility (98, 183) were used, despite of ages, to identify whether the bull was pubertal or postpubertal, and grouped under group A (n=5) or group B (n=7), respectively, (Table 4.1).

4.2.2. Hormone assay

The plasma LH concentrations were measured by a double-antibody radio-immunoassay (RIA) and testosterone and E_2 were measured by double antibody enzyme-immunoassay (EIA) as described in Chapter 2.6. The intra-assay and inter-assay coefficients of variation for LH assay were 11% and 8%, respectively. The intra- and inter- assay coefficients of variation (CVs) were 4.7 and 8.7 for testosterone and 7.1 and 14.9 for E_2, respectively.

4.2.3 Statistical analysis

JMP 5.0.1 software (SAS, Japan) was used for statistical analysis and the probability values $P<0.05$ were considered as significant. Data for age, body weight, SC, semen parameters and hormone concentrations were pooled and analyzed by ANOVA. Repeated measure analysis with univariate test was applied to analyze the time-group effect and their interactions on the changes of mean hormonal concentrations during 6 weeks. Student's *t*-test was applied to compare the means within and between two

groups. The pair-wise correlations by group were determined using multivariate analysis.

4.3. Results

The body weight, SC and semen parameters of volume, spermatozoa concentration, MAC and MAT were significantly higher ($P<0.01$) for group B (postpubertal bulls) as compared to group A (pubertal bulls) (Table 4.1). The mean concentrations of peripheral LH, testosterone and E_2 during 6 weeks of sampling period are shown in Fig. 4.1. The high variations between the individual animals, a non-uniform changing pattern and the similar levels of these hormones were common in both groups. Moreover, a considerable quantity of circulating E_2 (about or >1.0 pg/ml) was measured in these bulls. The repeated measure analysis of these hormones did not show significant effect for LH and testosterone. However, a significant ($P<0.05$) time effect and the time-group interaction on repeated measure analysis and significant differences within and between the groups in some weeks were observed for E_2.

The pair-wise correlations among the age, body weight, scrotal circumference, semen parameters and hormone concentrations by group are summarized in Table 4.2. Both groups demonstrated significant ($P<0.01$) positive correlations among the age, body weight and SC. Group A demonstrated significant positive correlations of the age with semen volume ($P<0.05$), MAC ($P<0.05$) and LH ($P<0.01$), and also of the semen volume with spermatozoa concentration ($P<0.05$), MAC ($P<0.01$) and LH ($P<0.01$). Similarly, this group showed a significantly positive correlation ($P<0.01$) between the body weight and LH, the SC and MAC, the spermatozoa concentration and MAC, the MAC and LH, the LH and E_2 and the testosterone and E_2. On the other hand, group B

demonstrated significant positive correlations of the body weight with semen volume (p<0.05) and spermatozoa concentration (P<0.01) and between the age and spermatozoa concentration (p<0.01), the SC and spermatozoa concentration (P<0.05), the MAC and MAT (P<0.01), the MAC and E_2 (P<0.05) and the MAT and E_2 (P<0.01). Moreover, this group also showed a significant negative correlation between the age and MAT (P<0.05) and the spermatozoa concentration and MAT (P<0.01). These results indicated the positive correlations of SC with the age and body weight in both groups, and also with the MAC in pubertal and spermatozoa concentration in postpubertal bulls. Moreover, LH correlated positively with the age, body weight, semen volume, MAC and E_2 in pubertal bulls. Interestingly, spermatozoa motility in postpubertal bulls correlated positively with E_2.

4.4. Discussion

The significant increase of SC in postpubertal bulls as compared to pubertal bulls observed in the present study has supported the previous report that SC continues to increase for some time after puberty (46). Similarly, the semen parameters of postpubertal bulls have also increased significantly as compared to pubertal bulls, which is consistent with the previous reports that semen quality in yearling bulls improves with age (6, 140). The present finding of positive correlation of SC with spermatozoa concentration in postpubertal bulls may indicate the importance of SC measurement to know the spermatozoa producing ability in bulls after puberty.

The high variation between the individual animals, a non-uniform changing pattern and the similar levels of LH, testosterone and E_2 in both groups made it difficult to suggest the exact physiological roles of these hormones. Therefore, the specific

results could not be drawn from the significant differences in the mean concentrations of E_2 within and between the groups observed during some weeks, despite of its time effect and time-group interactions. However, a considerable quantity of circulating E_2 was measured in bulls around and after puberty, which may have a significant physiological role. The positive correlations of LH concentrations with age, body weight, semen volume and MAC in pubertal bulls observed in the present study may indicate that LH may be important for early semen quality in bulls. Noteworthy of the present results was no significant correlations of testosterone with the age, body weight, SC, semen parameters and LH in both groups. Crucial cellular and structural changes in the seminiferous tubules take place prior to pubertal development in bulls from around 5 to 8 months of age (53, 54). The functional role of the transient rise of LH at around 3 months of age, followed by the testosterone rise has been considered important during prepubertal sexual development of bulls to trigger and support the cellular changes in the testis prior to puberty (7, 9, 13, 41). It can be suggested that testosterone may be important only up to certain threshold level, and beyond this level, testosterone concentration may not correlate with SC and semen quality around and after puberty.

Recent studies in several animal species have increasingly shown the important role of estrogen in male reproductive functions. Estrogen is produced by the irreversible aromatization of testosterone by cytochrome P450 aromatase (P450arom) enzyme. P450arom localization was reported in the Leydig cells of the ram (158) and boar (44), and in the Leydig cells and germ cells of the bear (175), rat (37), mouse (89, 128), human (39, 102) and rhesus monkey (138) testis. Studies based on the aromatase knockout (ArKO) male mouse has showed normal spermatogenesis at the beginning of puberty and decreased fertility with aging (130, 151), which may indicate the role of

estrogen in the functioning of more adult testis. Recent experiment in our laboratory has indicated the absence of immunohistochemical expression of P450arom in the 8-month-old bovine testis and its positive expression in the Leydig cells of 18-month-old bovine testis (unpublished). It can be speculated that P450arom activity starts in the bovine testis around puberty that follows the production of estrogen by the testis.

Although it is not yet understood in the bull testis, the localization of estrogen receptors has been reported in the Leydig cells, peritubular cells, and germ cells, as well as importantly, in the epithelial cells of the rete testis, efferent ducts and epididymus of the testis in several other species (78, 81, 130). Moreover, estrogen is reported to be important for human spermatogenesis (130). Efferent ductules are a major site for estrogen function in the male reproductive tract, which is responsible for reabsorption of rete fluid, and epididymus is also under the influence of estrogen (78). Transit of sperm through the efferent ductules and the epididymus is associated with significant maturational changes, such as gaining the capacity for progressive motility, and the most important functional changes in sperm occur in efferent ductules and head of the epididymus (19). The present result of a positive correlation between spermatozoa motility and E_2 levels in postpubertal bulls may indicate that bulls start to respond with this hormone after puberty.

A decrease of sperm motility has been reported in ArKO mice as well as in a man with an aromatase deficiency (38). It was reported that aromatase could be involved in the acquisition of sperm motility in human (102). On the basis of the specific functional regulation of efferent ductules and epididymus by estrogen in other animal species, the similar function of estrogen can be speculated in the bovine testis as well, which may contribute during the maturational process and, in turn, regulate the

motility of spermatozoa *in vivo*. Further studies on the activity of estrogen receptors and aromatase enzyme in the bovine testis around and after puberty are needed to better understand the functional role of estrogen. Although the present result is based on small number of animals monitored for short period, it is the first study of its kind, which may help to shed light on the role of estrogen in spermatozoa motility in male animals.

Table 4.1. Age, body weight, scrotal circumference (SC) and semen parameters in the peripubertal (group A, n=5) and postpubertal (group B, n=7) bulls.

	Group A	Group B
Age (month)	10.2 ± 0.6	14.1 ± 0.8
Body weight (kg)	319.0 ± 20.89 [a]	424.7 ± 18.22 [b]
Scrotal circumference (cm)	26.9 ± 1.6 [a]	33.2 ± 0.5 [b]
Semen volume (ml)	2.4 ± 0.4 [a]	6.6 ± 0.3 [b]
Spermatozoa concentration (x10^6/ml)	34.0 ± 9.0 [a]	847.0 ± 59.0 [b]
Progressive motility after collection (+++%)	15.9 ± 3.8 [a]	69.6 ± 0.7 [b]
Progressive motility after freezing and thawing (+++%)	ND	34.0 ± 0.9

ND: not determined

Values are expressed as Mean ± SEM.

Different superscripts between the groups denote significant differences ($P<0.01$).

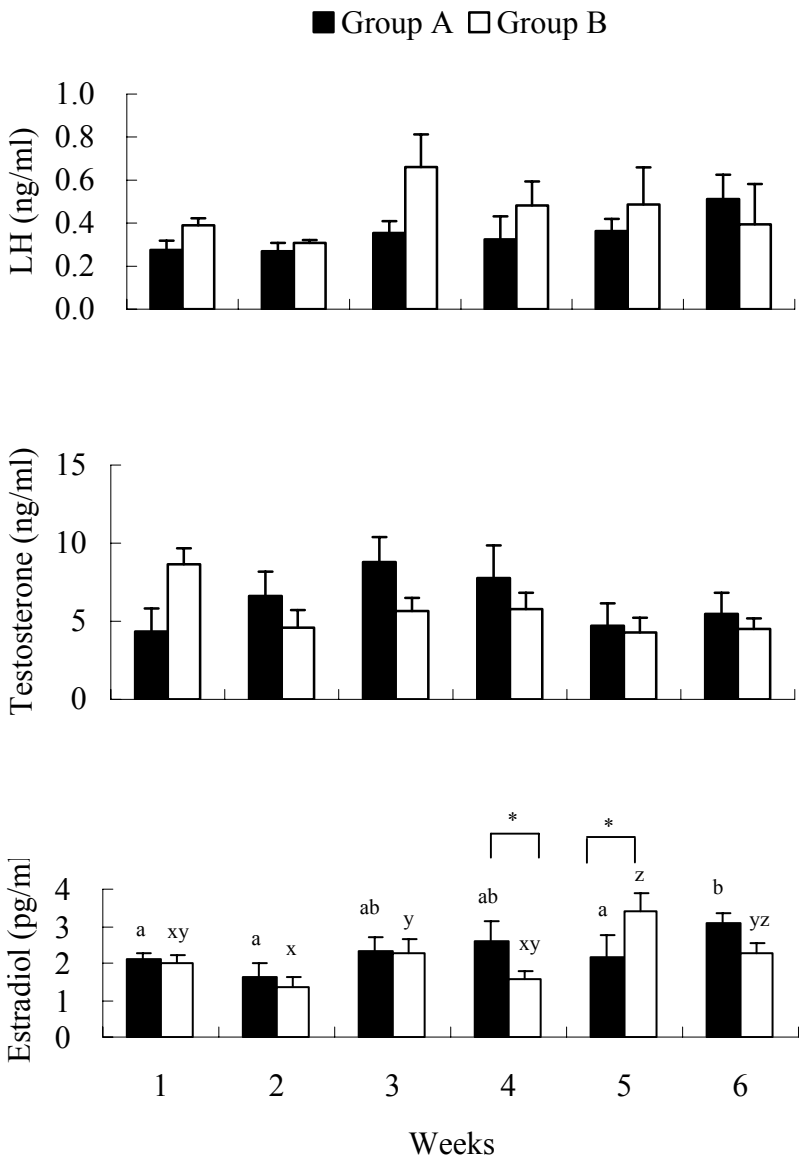


Fig. 4.1. Mean concentrations of LH, testosterone and estradiol in group A (pubertal bulls) and group B (postpubertal bulls). Samples were collected once a week for 6 weeks. The comparison of means are shown only for estradiol since it showed a significant time effect as well as the interaction between time and group on repeated measure analysis. Different superscripts within the group and stars (*) between the groups denote significant difference ($P<0.05$).

Table 4.2. The pair-wise correlations among the age, body weight, scrotal circumference, semen parameters and endocrine parameters by groups (group A: peripubertal bulls, n=5 and group B: postpubertal bulls, n=7).

	Group	Body weight	SC	Semen volume	Spermatozoa concentration	MAC	MAT	LH	Testosterone	Estrogen
Age (month)	A	0.988 [a]	0.774 [a]	0.392 [b]	0.164	0.406 [b]	ND	0.514 [a]	0.239	0.198
	B	0.929 [a]	0.600 [a]	0.230	0.621 [a]	-0.028	-0.412 [b]	-0.207	0.132	-0.183
Body weight	A		0.759 [a]	0.282	0.116	0.324	ND	0.452 [a]	0.217	0.175
	B		0.454 [a]	0.359 [b]	0.457 [a]	0.002	-0.342	-0.201	0.206	-0.230
SC	A			0.225	0.321	0.449 [a]	ND	0.305	0.073	0.159
	B			0.230	0.355 [b]	-0.159	-0.240	-0.082	0.021	0.115
Semen volume	A				0.401 [b]	0.677 [a]	ND	0.518 [a]	0.138	0.283
	B				0.160	-0.107	-0.107	0.055	0.248	0.132
Spermatozoa concentration	A					0.716 [a]	ND	0.159	0.256	0.297
	B					-0.087	-0.463 [a]	0.019	0.045	-0.154
MAC	A						ND	0.549 [a]	0.097	0.283
	B						0.491 [a]	-0.154	-0.179	0.339 [b]
MAT	A							ND	ND	ND
	B							-0.126	-0.062	0.457 [a]
LH	A								0.208	0.470 [a]
	B								-0.059	0.273
Testosterone	A									0.571 [a]
	B									0.109

SC: Scrotal circumference, MAC: motility of spermatozoa immediately after semen collection, MAT: motility of spermatozoa after freezing and thawing of semen, ND: not determined. The values with the superscripts, a ($P<0.01$), and b ($P<0.05$), showed a significant correlation.

Chapter 5

Cellular, Some Cytoskeletal and Functional Alterations in the Spontaneous Cryptorchid Testis

Chapter 5.A

Alterations in the histological and immunohistochemical localization patterns of α-smooth muscle actin (SMA), vimentin and cytokeratin

5.A.1. Introduction

Cryptorchidism is the failure of testis to descend into the scrotum, exposing it to body heat that severely affects normal spermatogenesis. Previous studies have indicated that locally induced heat or cryptorchidism not only causes germ cell loss, but also impairs the structures and functions of the basement membrane, peritubular cells, and Sertoli cells (73, 74, 87, 97). Cytoskeletal proteins provide structural and functional support to cells. Study of cytoskeletal proteins, such as α-smooth muscle actin (SMA) and vimentin, that are localized in the seminiferous peritubular cells and Sertoli cells, respectively (54), may be useful for understanding the changes in these cells.

Seminiferous peritubular and mediastinal α-SMA appears around 4 months of age, and the Sertoli cell vimentin may play a key role in stabilizing the Sertoli cell nuclei during transformation and maturation in bovine testes (54). A remarkable structural disruption of actin filaments in the rat testis (108) and alteration and disruption of Sertoli cell vimentin in the rat (179) and rhesus monkey (186) have been reported after experimental cryptorchidism. Cryptorchid testes generally reveal different states of Sertoli cell immaturity (147) and demonstrate co-expression of vimentin, desmin, and cytokeratins (153, 186). Moreover, the expression of cytokeratin in the adult seminiferous epithelium has been regarded as a sign of either maintaining or regaining undifferentiated immature feature in the testis of human with impaired fertility (15, 22, 114, 153, 167, 168, 171) and spontaneous cryptorchidism (153), and in

rhesus monkey after experimental cryptorchidism (186). However, the cytoskeletal alterations in the cryptorchid testis as compared to the normal testis during postnatal development are not yet understood. The objectives of the present study were to immunohistochemically localize α-SMA and vimentin, and compare their localization patterns and cellular development between the cryptorchid and contralateral scrotal testes of postnatally developing unilateral cryptorchid bulls. The present results clearly demonstrate the cellular immaturity and alterations in the localization patterns of α-SMA and vimentin in bovine testes continuously exposed to body heat during postnatal development.

5.A.2. Materials and Methods

Testes samples were collected after slaughter or surgical castration from postnatally growing spontaneous unilateral cryptorchid bulls (n=10) reared at Obihiro University and various farms in Hokkaido, Japan. The recorded ages of the bulls were 1 month (n=3), 3 months (n=1), 5 months (n=3), 8 months (n=2) and 18 months (n=1). All animals were Holstein-Frisian bulls, except for one Japanese Black bull (8 months). The positions of the cryptorchid testes were abdominal as shown in Fig. 5.A.1A (n=4) and inguinal at the level of the scrotal base (n=6). The contralateral scrotal testes (n=8) were also collected at the same time and used as controls. Fetal testis samples collected from bovine fetuses (n=2, CRL=8.9 and 46.0 cm) were used as positive controls for cytokeratin immunostaining.

The collection of testis samples, their histological preparations and staining with hematoxylin-eosin (HE) stain and immunohistochemical technique with α-SMA, vimentin and cytokeratin was performed as explained in Chapter 2.3, 2.4 and 2.5. The

primary antibodies used were the mouse monoclonal anti-α-smooth muscle actin (1:5000; Clone No. 1A4, Sigma Chemical Co., St. Louis, USA), polyclonal anti-calf lens vimentin (1:150; MEDAC, Hamburg, Germany) and the rabbit polyclonal anti-keratin (1:100, DAKO, Copenhagen, Denmark). The control sections were treated with normal horse and normal goat serum instead of α-SMA and vimentin or cytokeratin primary antisera, respectively. Finally, the sections were counterstained with Mayer's hematoxylin solution.

5.A.3. Results

In comparison with the control testes, the cryptorchid testes were hypoplastic, and the degree of hypoplasia increased with advancing age (Table 5.A.1, Fig. 5.A.1B).

5.A.3.1. Cellular alterations in the postnatally growing cryptorchid testes

Almost no cellular alterations were observed until 3 months of age in the cryptorchid testes, when compared with the contralateral control testes. However, the cryptorchid testes at 5 months of age and onwards, revealed considerable histopathological changes that increased with advancing age. Apart from a rapid increase in diameter of the seminiferous tubules, the transformation process of the Sertoli cells in the control testes was indicated by nuclei arranged in multiple layers in 5-month-old testes (Fig. 5.A.2A), which underwent the process of maturation and positioning basally in the 8-month-old testes (Fig. 5.A.2C). On the other hand, the Sertoli cells in the 5-month-old cryptorchid testes showed a highly immature appearance with a single layer of basally packed round nuclei (Fig. 5.A.2B), which was seen in multiple layers in the 8-month-old cryptorchid testes (Fig. 5.A.2D). Initiation

and advancement of spermatogenesis were progressive in the control testes at 5 months (Fig. 5.A.2A), 8 months (Fig. 5.A.2C), and 18 months (Fig. 5.A.2E) of age, whereas it was almost absent in the cryptorchid testes during the same periods (Figs. 5.A.2B, D, and F). Moreover, the cryptorchid testis at 18 months of age (Fig. 5.A.2F) demonstrated a characteristic histopathological alteration of the seminiferous tubules, mostly exhibiting tubules with only Sertoli cells and immature Sertoli cells with elongated or round nuclei in a pseudostratified disposition.

5.A.3.2. Alterations in the localization patterns of α-SMA and vimentin in the cryptorchid testes

The localization patterns of α-SMA and vimentin did not reveal a considerable difference between the cryptorchid and contralateral control testes until 3 months of age, except for moderately increased perinuclear vimentin in the Sertoli cells of 3-month-old control testis, which was not noticed in the cryptorchid testis. Similarly, weak expression of α-SMA in the sub epithelial and stromal cells of the straight tubules and rete testis at 5 months of age did not improve until 18 months of age in the cryptorchid testes.

No α-SMA appeared in cells other than the blood vessels until 3 months of age in both the cryptorchid and control testes. The control testes from 5-month-old (Fig. 5.A.3A) and 8-month-old (Fig. 5.A.3C) bulls demonstrated continuous seminiferous peritubular α-SMA. Peritubular α-SMA did not appear in the cryptorchid testes at 5 months (Fig. 5.A.3B) and 8 months (Fig. 5.A.3D) of age, except for very weak intermittent α-SMA in a few of the relatively larger seminiferous tubules in the 8-month-old testes (Fig. 5.A.3D). However, almost the same pattern was observed in the

18-month-old control (Fig. 5.A.3E) and cryptorchid testes (Fig. 5.A.3F). These results clearly indicated that testicular myoid cell differentiation was considerably delayed in the postnatally growing cryptorchid testes.

In vimentin immunoreactions, almost the same patterns were observed in the positive cells of the vessel walls, interstitium, seminiferous peritubules, and basal epithelia of the straight tubules and rete testis of the cryptorchid and contralateral control testes. However, the Sertoli cell vimentin demonstrated considerable alterations from 5 months of age and onwards in the cryptorchid testes. The control testes at 5 months of age (Fig. 5.A.4A) demonstrated a transforming pattern with prominent infranuclear vimentin extensions connecting the Sertoli cell nuclei to the basal membrane, while the cryptorchid testes demonstrated a highly immature pattern of very weak and basal perinuclear Sertoli cell vimentin (Fig. 5.A.4B). The control testis at 8 months of age (Fig. 5.A.4C) demonstrated a normal pattern of shortening of the infranuclear vimentin extensions, indicating that basal positioning of the Sertoli cell nuclei was in progress, while the cryptorchid testis demonstrated infranuclear vimentin extensions with a pattern that was similar to a transforming pattern (Fig. 5.A.4D). At 18 months of age, the control testis (Fig. 5.A.4E) demonstrated a mature pattern of perinuclear Sertoli cell vimentin, while the cryptorchid testis still demonstrated a transforming pattern, although considerable weakening of the Sertoli cell vimentin extensions was observed (Fig. 5.A.4F). This indicated that there was a considerable delay in the Sertoli cell vimentin in attaining the transformation pattern in postnatally developing bovine cryptorchid testes. Moreover, the transformation pattern of vimentin was prolonged for several months without attaining a mature pattern, and weakened in intensity as the animal matured.

5.A.3.3. Immunohistochemical expressions of cytokeratin in the spontaneous cryptorchid bovine testes

Cytokeratin was expressed in the Sertoli cells of CRL=8.9 cm fetal testis (Fig. 5.A.5A), while it was absent in the CRL=46.0 cm fetal testis (Fig. 5.A.5B) as well as in the 1-month-old (Fig. 5.A.5C), 8-month-old (Fig. 5.A.5D), and 18-month-old (Fig. 5.A.5E) cryptorchid testes. However, cytokeratin was expressed in the epithelia of rete testis of the fetal (Fig. 5.A.5B), cryptorchid (Figs. 5.A.5C, D, E) bull testes. The positive expressions of cytokeratin in the Sertoli cells of CRL=8.9 cm fetal testis and in the rete testis epithelia of other testes were used as positive controls. This result indicates that cytokeratin expression in the bovine Sertoli cell disappears in between CRL=8.9 cm to CRL=46.0 cm fetal testis, and it is not expressed in the cryptorchid bovine testis until 18 months of age.

5.A.4. Discussion

The histological observations of the normal control testes in this study were generally consistent with previous reports in the bovine testis during postnatal development (48, 163). The immunohistochemical localization of α-SMA and vimentin in the control testes in the present study followed the normal postnatal pattern described previously (54).

Different histological patterns of Sertoli cell immaturity have been reported in the human postpubertal cryptorchid testis (147), and the immature Sertoli cells of the cryptorchid rhesus monkey (186) and human (153) have demonstrated co-expression of vimentin, desmin, and cytokeratins. It has been suggested that local heat *in vivo* affects the fine structures of the Sertoli cells and the basement membrane of the seminiferous

tubules of the rat testis (87). Similarly, it has also been reported that induction of experimental cryptorchidism in rats impairs the morphology and function of the Sertoli cells and peritubular tissue (73, 74, 97). Although, the structural actin and α-SMA isomers may differ in their heat sensitivities, the structural actin filaments in the rat testes were remarkably disrupted after 7 days of experimental cryptorchidism (108). The present results support the facts that cryptorchidism causes alterations in testes morphology, impairs spermatogenesis, and also contributes to seminiferous tubule and Sertoli cell immaturity. Moreover, the present study is the first of its kind to demonstrate the relationship between cellular immaturity and failure of the spermatogenic process with cytoskeletal changes, such as delayed myoid cell differentiation and delayed attainment of the transformation pattern of the Sertoli cell vimentin, which failed to attain a mature pattern in the postnatally developing bovine testes under continuous exposure to body heat. The present results further demonstrated that the vimentin localization pattern in the bovine Sertoli cells might indicate their maturity. The α-SMA in the rete testis may contribute to tubular contractility and spermatozoa transportation (54). The failure of the α-SMA to improve in the rete testis of the bovine cryptorchid testes until 18 months of age may reduce its contractility.

Although alteration and disruption of the Sertoli cell vimentin filaments have been reported in rats (179) and rhesus monkeys (186) after experimental cryptorchidism, the present data did not reveal such disruptions until 18 months of age in the bovine cryptorchid testis. However, a considerable weakening of the vimentin extensions in the cryptorchid testis was observed as the age of the animal increased. This weakening of the vimentin filaments may ultimately lead to disruption of the vimentin as the animal continues to age. As vimentin may play a key role in stabilizing bovine Sertoli cell

nuclei during the maturation process and may ultimately help to position them basally (54), it can be speculated that weakening of the vimentin filaments may cause a functional failure that may interfere with the maturation process of the Sertoli cells.

It has been reported that the appearance of peritubular α-SMA was induced by testosterone in the monkeys (157). It was previously reported that the appearance of α-SMA in the bovine testis (54) and postnatal initiation of spermatogenic cell division around 4 months of age might be related to an increased testosterone activity around that age (9, 48). In long term bilaterally cryptorchid rams, testosterone concentrations in the peripheral and spermatic veins were found to be normal (106), but in rats made unilaterally cryptorchid at birth, androgen concentrations in the cryptorchid testes were much lower than in scrotal testes (20). Based on the present experimental model of direct comparison between the cryptorchid and contralateral normal testes of the same animal, it can be suggested that the alterations observed in the cryptorchid testes here is more related to continuous thermal stress than the effects of testosterone, although the differences in androgen concentrations, the paracrine androgenic effect, and differences in receptor distributions between cryptorchid and scrotal testes in the bovine are not fully understood. Further study may be required to discover, if any downregulation in the expression of a specific α-SMA gene or its mRNA level is a possible cause of its delayed appearance in the bovine cryptorchid testis during postnatal development. It is also important to investigate the relationships of cellular, structural, and functional changes in the bovine testes under experimentally induced thermal stress *in vivo*.

Cytokeratin is expressed in the adult seminiferous tubules of testis with spontaneous cryptorchidism in human (153) and experimental cryptorchidism in rats (186), as a sign of undifferentiated immature Sertoli cells. However, interestingly,

cytokeratin was not expressed in the bovine cryptorchid testes. It may be due to species-specific characters of cytokeratin expression. This striking finding may indicate that vimentin expression pattern, not the cytokeratin, can be a reliable indicator of the differentiated Sertoli cells, capable of supporting spermatogenesis in the bovine testis. It can be concluded that the alterations of α-SMA and vimentin observed here in postnatally developing bovine cryptorchid testes might be related to the effect of continuous exposure to body heat interfering normal maturity.

Table 5.A.1. Comparison of testes measurements between the cryptorchid and contralateral scrotal (control) testes of unilateral cryptorchid bulls at different ages.

Age (Animals, n=)	Testes measurements							
	Length (cm)		Breadth (cm)		Width (cm)		Weight (g)	
	C	Cr	C	Cr	C	Cr	C	Cr
1 month (2)	3.5, 3.5	3.0, 3.2	2.0, 2.0	2.2, 2.0	1.6, 1.5	1.6, 1.6	4, 4	4, 4
3 months (1)	4.5	4.0	2.5	2.4	1.5	1.5	15	10
5 months (1)	5.5	4.2	3.5	2.5	2.5	2.0	32	18
8 months (1)	9.0	5.5	5.0	3.2	3.5	2.4	115	30
18 months (1)	10.0	6.5	6.5	4.5	4.0	2.5	200	50

C: Contralateral normal scrotal testis used as a control, Cr: Cryptorchid testis

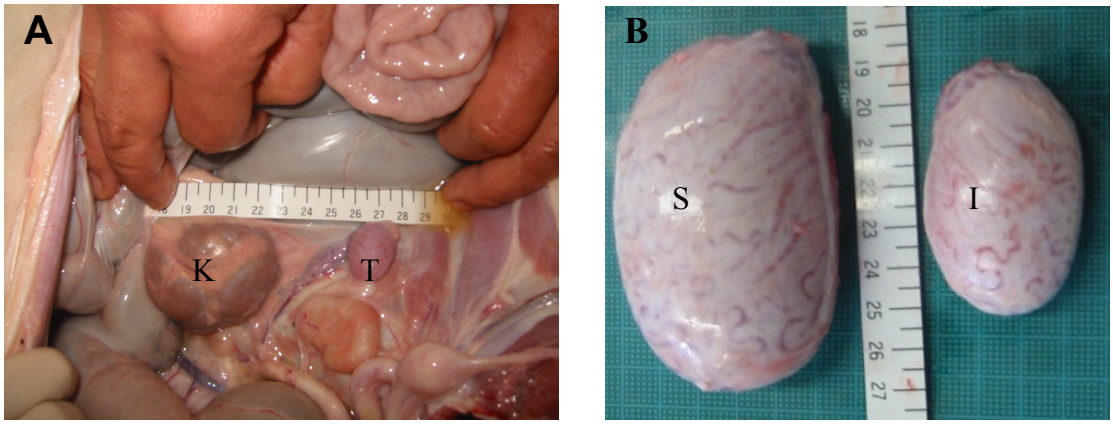


Fig. 5.A.1. Anatomical location of the right testis in 1-month-old unilateral cryptorchid bull, which was located close to the kidney in the abdominal cavity (K: kidney, T: testis). B. Comparison of the scrotal testis (S) and inguinal testis (I) in the 8-month-old bull after removal of the epididymus.

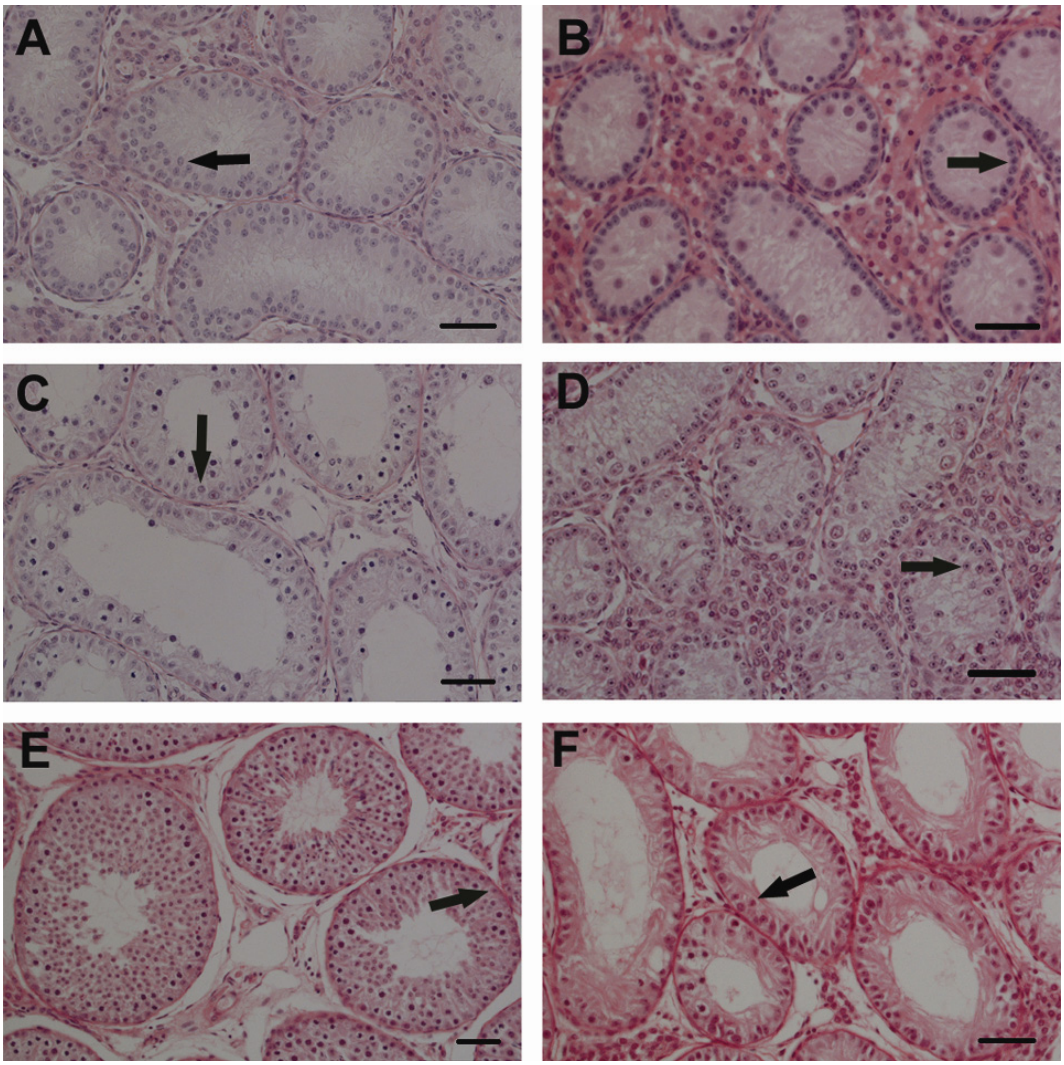


Fig. 5.A.2. Hematoxylin eosin (HE) staining of sections of paired testes from representative unilateral cryptorchid bulls. All arrows indicate Sertoli cell nuclei. Control scrotal testes sections at 5 months (A), 8 months (C), and 18 months (E) of age showed normal maturational changes in the Sertoli cells and progressive spermatogenesis. Cryptorchid testes at 5 months of age (B) showed highly immature Sertoli cells with a basal arrangement, and at 8 months of age (D), showed Sertoli cells under the maturation process. The cryptorchid testis from an 18-month-old bull (F) showed mostly Sertoli-cell-only seminiferous tubules with a pseudostratified disposition of immature Sertoli cell nuclei. Bars: 50μm.

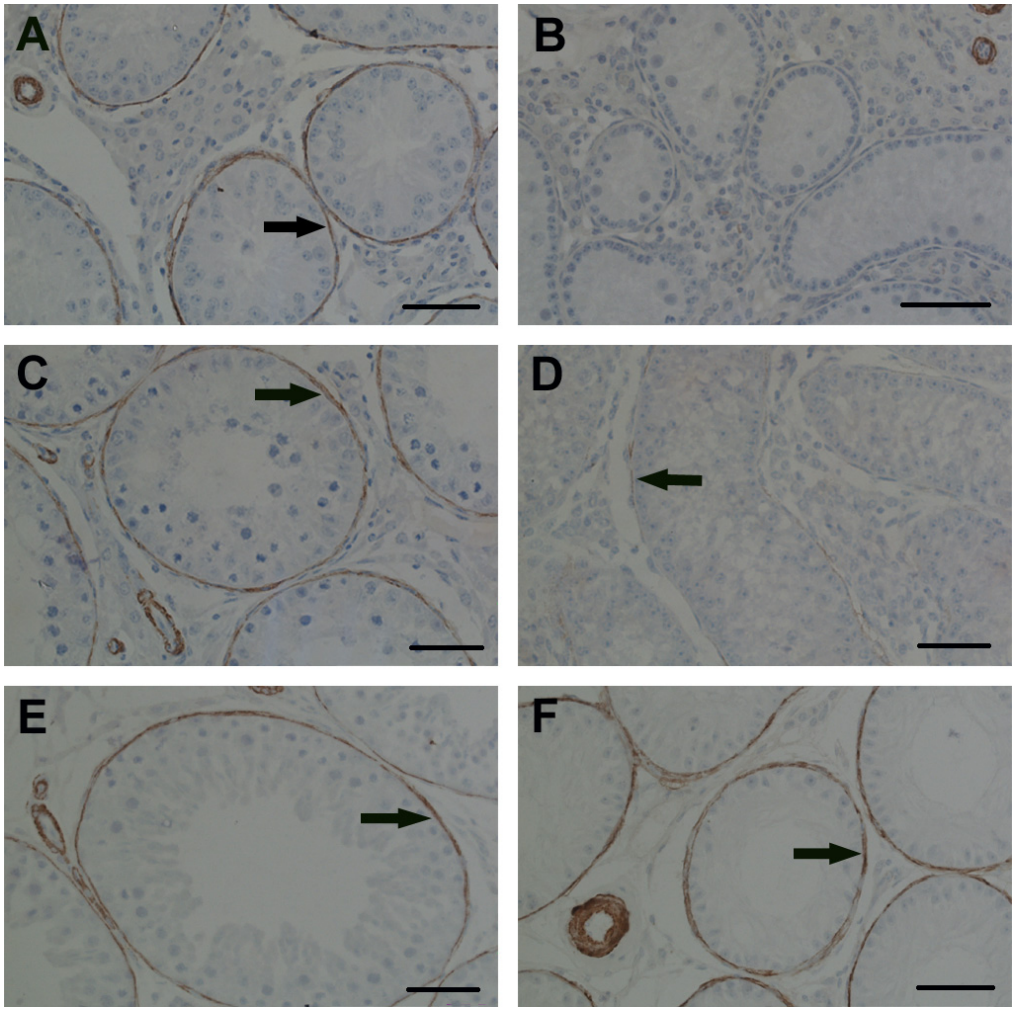


Figure 5.A.3. Alpha smooth muscle actin (SMA) immunoreactions of paired testes from representative unilateral cryptorchid bulls. All arrows indicate seminiferous peritubular α-SMA. Control scrotal testes sections at 5 months (A), 8 months (C), and 18 months (E) of age and a contralateral cryptorchid testis at 18 months (F) of age showed the continuous seminiferous peritubular α-SMA. Cryptorchid testes at 5 months (B) and 8 months (D) of age showed the absence of seminiferous peritubular α-SMA, except for some relatively larger tubules with very weak intermittent α-SMA in the 8-month-old cryptorchid testis. Bars: 50 μm.

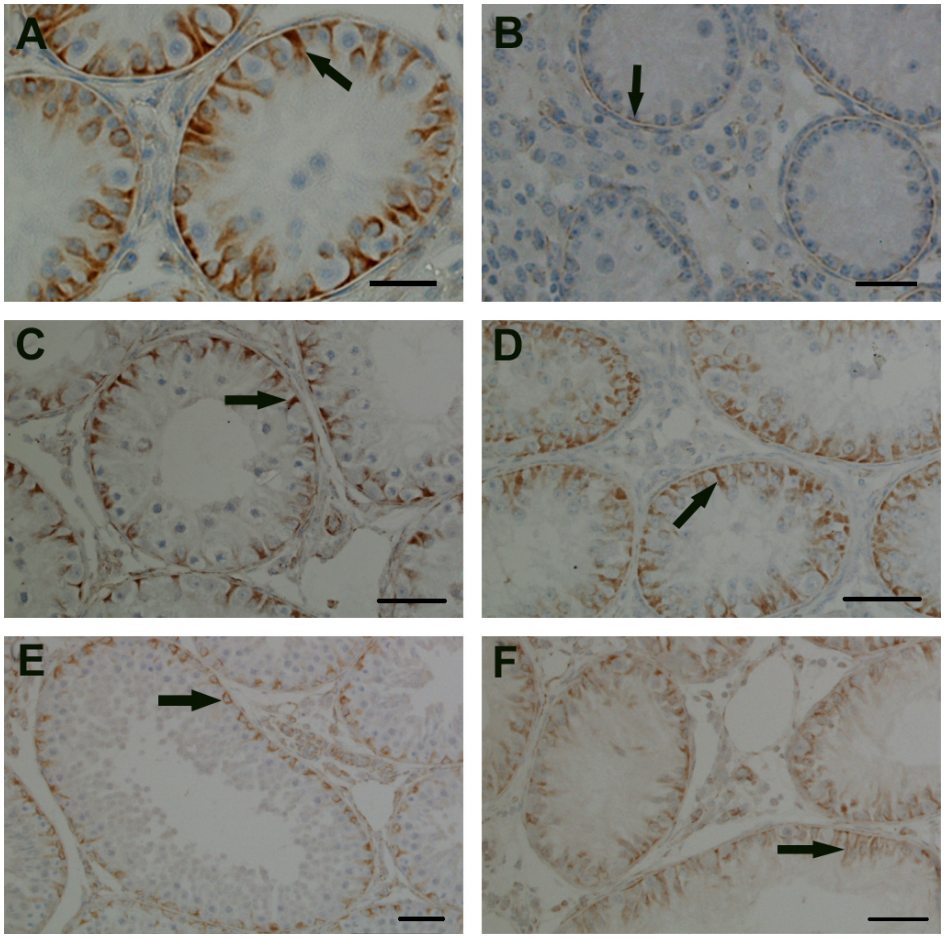


Figure 5.A.4. Vimentin immunoreactions of paired testes from representative unilateral cryptorchid bulls. All arrows indicate Sertoli cell vimentin. Control scrotal testis sections at 5 months of age (A) showed a transforming pattern for the Sertoli cell vimentin with characteristic infranuclear extensions, at 8 months of age (C), they showed almost the same pattern with a relative shortening of the vimentin extensions, and at 18 months of age (E), they showed a mature pattern. Contralateral cryptorchid testes at 5 months of age (B) showed a highly immature pattern with very weak and basal Sertoli cell perinuclear vimentin, and at 8 months (D) and 18 months (F) of age, they showed a similar pattern to the transforming pattern, but with a considerable number of weak vimentin extensions at 18 months of age (F). Bars: A and B 25 μm, C, D, E, and F 50 μm.

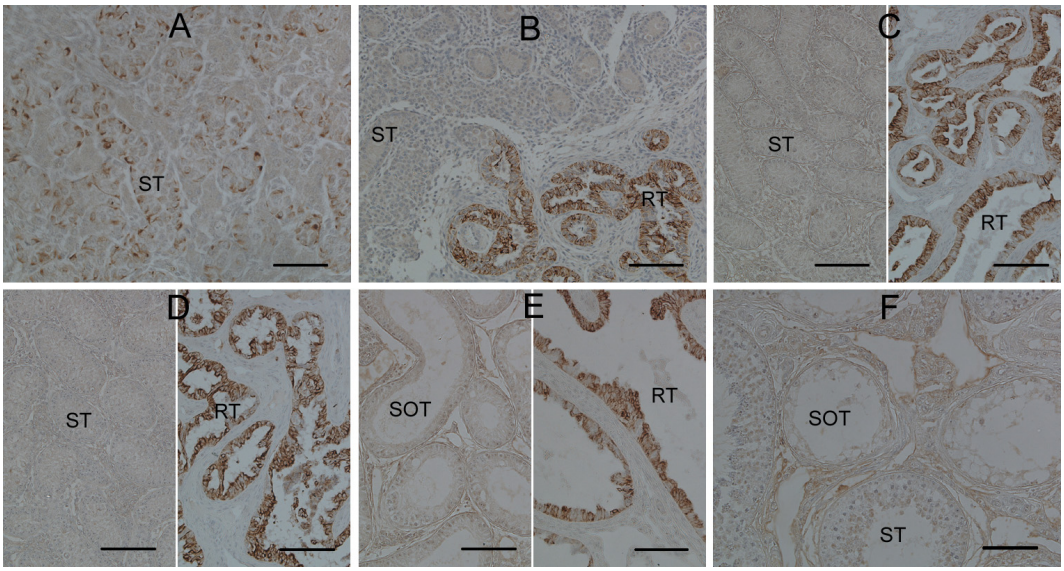


Fig. 5.A.5. Immunohistochemical expression patterns of cytokeratin in the bovine fetal (CRL=8.9 cm fetus: A, CRL 46.0 cm fetus: B), cryptorchid (C, D and E) and mixed atrophied (F) testes. ST: seminiferous tubule, SOT: Sertoli cell only tubule, RT: rete testis. Bars: 100 μm.

Chapter 5.B

Alterations of the steroidogenic functions

5.B.1. Introduction

Cryptorchid testes reveal the cellular and structural immaturity of the seminiferous tubules (54). Temperature affects several Leydig cells organelles associated with steroidogenesis (59, 96). Similarly, cryptorchid testis in the mouse secretes more estradiol (E_2) and less testosterone (23). However, such changes are not investigated in bulls.

Several enzymes involved in testosterone synthesis pathway are heat sensitive and their activities are reduced in the cryptorchid testis, possibly causing reduced testosterone secretion (20, 60, 83, 88, 120). The biosynthesis of steroid hormones begins with the enzymatic cleavage of side chain of the substrate of cholesterol to form pregnenolone (135), which is catalyzed by the cytochrome P450 side chain cleavage (P450scc) enzyme. This is the first and rate-limiting step in steroid biosynthesis. Cascades of several enzymatic conversions follow during the synthesis of testosterone from pregnenolone. Cytochrome P450 aromatase (P450arom) is the enzyme responsible for the conversion of androgens into estrogens. In the bovine testis, the cellular localization of these enzymes is not fully understood and the elucidation of their expression pattern under cryptorchid condition during postnatal development may further provide new insights in the alteration of steroidogenic functions under heat stress during sexual development. Moreover, cryptorchidism in rat (49, 148) and raised testicular temperature in ram (121) is shown to reduce the responsiveness with human chorionic gonadotrophin (hCG) hormone to stimulate testosterone synthesis. However,

such effects in bulls are so far not understood, eventhough testicular functions in bulls under heat stress are always the big concerns. The objectives of the present study were to investigate the alterations of steroidogenic functions in the postnatally developing bovine testis exposed continuously to increased heat by using the cryptorchid testes as models.

5.B.2. Materials and methods

Experiment 1.

Testes samples were collected after slaughter or surgical castration from postnatally growing spontaneous unilateral cryptorchid bulls (n=10) explained in Chapter 5.A.2, and were used for the present study, too. The contralateral scrotal testes (n=8) were used as control against the cryptorchid testes. The collection of testis samples and preparation for immunohistochemical study is explained in chapter 2.3 and 2.5, respectively. Immunohistochemical staining was performed for P450scc and P450arom enzymes and compared the expression patterns between the control and cryptorchid testes. The primary antibodies used were the polyclonal antibodies in rabbit, cytochrome P450 side chain cleavage (1:200, CHEMICON International, Inc., CA 92590, USA) and cytochrome P450 aromatase (Prof. Toshio Tsubota, Gifu University, Japan). The control sections were treated with normal sera instead of the primary antisera. Finally, the sections were counterstained with Mayer's hematoxylin solution.

Experiment 2.

Unilateral cryptorchid bulls (n=3, age: 7.3 ± 1.2 month) were castrated to remove the scrotal testis keeping cryptorchid testis intact. These bulls were later treated

with hCG (2000-5000 IU IM) and the peripheral blood samples were collected as 0 (treatment day), 4, 5 and 7 days, plasma was harvested and later measured for testosterone concentration in order to investigate the response of hCG on the production of testosterone by the cryptorchid testes. Furthermore, in order to compare the testosterone and E_2 production capacity by the cryptorchid testis, one 1-month-old unilateral cryptorchid bull was bilaterally castrated and another 1-month-old unilateral cryptorchid bull was unilaterally castrated to remove the scrotal testis keeping its cryptorchid testis intact. From both bulls, peripheral blood was collected daily as 0 (castration day), 1, 2, 3, 4 and 5 days. Plasma was harvested and assayed for LH (RIA), testosterone (EIA) and E_2 (EIA), as described in chapter 2.6.

5.B.3. Results

Experiment 1

The expression patterns of P450scc and P450arom and comparison between the contralateral scrotal (control) testis and the cryptorchid testis at different stages during postnatal development of unilateral cryptorchid bulls are demonstrated in Fig. 5.B.1 and 5.B.2, respectively.

P450scc was expressed only by the Leydig cells of both the control and cryptorchid testes from 1 month to 18 months of age (Fig. 5.B.1A-J). The weak expression by a few Leydig cells at 1 month of age (Fig. 5.B.1A, B) and a strong expression by majority of the Leydig cells at 3 months (Fig. 5.B.1C, D) and 5 months (Fig. 5.B.1E, F) of ages were almost similar between the control and cryptorchid testes. In the control testes at 8 months (Fig. 5.B.1G) and 18 months of age (Fig. 5.B.1I), a strong expression of P450scc by the majority of Leydig cells continued. However in the

cryptorchid testes, only a few Leydig cells expressed weak P450scc at 8 months (Fig. 5.B.1H) and 18 months (Fig. 5.B.1J) of age, and the progression of weak expression and the reduction of expressing Leydig cells were considerable as the animal matured.

P450arom was not expressed in the control testes at 1 month (Fig. 5.B.2A), 3 months (Fig. 5.B.2C), 5 months (Fig. 5.B.2E) and 8 months (Fig. 5.B.2G) of age. However, it is expressed by the Leydig cells at 18 months (Fig. 5.B.2I) of age, which may indicate that Leydig cells could be the source of estrogen in the mature bovine testes. Interestingly in the cryptorchid testes, weak P450arom expression was observed in the Sertoli cells at 1 month (Fig. 5.B.2B) of age, which gained its intensity in the 3-month-old (Fig. 5.B.2D) and 5-month-old (Fig. 5.B.2F) cryptorchid testes, and got considerably strong in the 8-month-old (Fig. 5.B.2H) and 18-month-old (Fig. 5.B.2J) cryptorchid testes. Moreover, the Leydig cells of 18-month-old cryptorchid testis also expressed P450arom, but the expression pattern was almost similar as that of the control testis at this age.

Experiment 2

The response of hCG treatment by the cryptorchid testis after removal of the scrotal testis in 3 bulls is shown in Fig. 5.B.3. This result clearly indicated a very low (0.4 ± 0.1 ng/ml, Mean ± SEM) basal level (day of treatment) of testosterone in cryptorchid testis only intact bulls. Moreover, there was no response of hCG treatment until 7 days post-treatment in these bulls, which further indicated the inability of cryptorchid testes to synthesize testosterone in response to hCG treatment.

The comparison of peripheral LH, testosterone and E_2 concentrations in 1-month-old unilateral cryptorchid bulls, after bilateral castration in one bull and

unilateral castration of the scrotal testis leaving the cryptorchid testis intact in another bull, is shown in 5.B.4. Bilaterally castrated bull had an increased LH concentration for the following 2 days, which thereafter returned to the concentration similar to 0 day. However, such changes in unilaterally castrated bull with intact cryptorchid testis were not observed. Interestingly, both bulls demonstrated rapid decrease in testosterone concentration after castration. Moreover, E_2 concentration in bilaterally castrated bull showed decreased tendency after castration, whereas, it was maintained in the unilateral castrated bull with intact cryptorchid testis.

5.B.4. Discussion

The control testes used in the present study were the contralateral scrotal testes of postnatally growing unilateral cryptorchid bulls, which generally exhibit similar postnatal developmental pattern as that of the normally developing testes (53, 54). The present study demonstrated the immunohistochemical localization of P450scc enzyme in the Leydig cells of both control and cryptorchid testes, which may indicate that Leydig cells are responsible for the enzymatic cleavage of side chain of the substrate of cholesterol in the bovine testis. The P450scc expressing Leydig cells were few in 1-month-old testes, while most of the Leydig cells expressed this enzyme at and after 3 months of age in the control testes. However, P450scc positive Leydig cells and the intensity of its expression in the cryptorchid testis decreased in 8-month-old and 18-month-old testes with more considerable change in the later age. The present finding of P450scc localization only in the Leydig cells of bovine testes is consistent with the previous findings in rat (136), Hokkaido sika deer (76) and Shiba goat (181). The activities of several other enzymes such as 17-α hydroxylase, 17β-hydroxysteroid

dehydrogenase (20), 3β-hydroxysteroid dehydrogenase (88) and 17,20-lyase (60) involved in the Δ^4 and Δ^5 testosterone biosynthetic pathways are reported to be heat sensitive. The present finding, for the first time, indicated the down-regulation of P450scc enzyme in the postnatally developing bovine cryptorchid testis after 5 months of age. It can be suggested that the prolonged exposure of the testis to body heat might have been the cause of such down-regulation.

The most striking finding of this study was the inability of cryptorchid testes to synthesize testosterone in response to hCG treatment. Increased testicular temperature due to cryptorchidism or local heating of the testis has been shown to reduce both testicular blood flow and testosterone secretion (49, 68) and to reduce the LH receptor content in the rat testis (85). The present finding of the downregulation of P450scc enzyme in the testis after prolonged exposure with the body heat may add one more possibility to explain the decreased responsiveness to hCG treatment by the testis under prolonged heat stress.

In the present study, P450arom was not localized in the control testis until 8 months of age, although a very weak expression in the Sertoli cells of some of the specimens of 1-month-old and 3-month-old testes was observed. However, the results were not uniform to indicate its activity and it was also not possible to rule out about the aromatization of testosterone in the Sertoli cells of young bovine testis. It was clear from the present result that the Leydig cells of mature bovine testis express P450arom, which is consistent with the reports in ram (158), boar (44), stallion (77), Sika deer (76) and Shiba goat (181). The expression sites of P450arom may have a wide variation between the species since its localization, apart from the Leydig cells, was reported in the Sertoli cells and germ cells in several other species (23, 37, 39, 89, 102, 128, 138,

175). It can be suggested that the Leydig cells also provide the site of aromatization and produce E_2 in the mature bulls, which may also indicate the role of E_2 in the functioning of the mature bovine testis.

Another striking finding of the present study was the P450arom expression by the Sertoli cells of the postnatally developing bovine cryptorchid testis, which was noticed from 1 month of age and increased considerably until 8 months of age. Rats made unilaterally cryptorchid at birth (20, 49) had higher concentration of estrogen receptors in the abdominal testis, but lower aromatase activity. Similarly, it was reported in the unilateral cryptorchid boar that lower aromatase activity was present in the Leydig cells of the abdominal testis (145). Moreover, unilateral cryptorchid boars and stallions showed impaired estrogen production compared with that of the contralateral scrotal testis (155). These results are in contrast with the present result and may indicate for the species difference. However, the relatively higher level of E_2 and lower level of testosterone in the one-month-old cryptorchid testis intact bull in the present study may indicate higher E_2 production by the cryptorchid testis as reported in the cryptorchid testis of rat (20, 49) and mouse (23). It can be speculated that more testosterone might have been utilized to synthesize E_2 in this bull causing less testosterone, which is comparable with the bilaterally castrated bull, despite of the similar P450scc enzyme activity observed between the cryptorchid and the contralateral scrotal testis at one month of age. Noteworthy in this finding is also the increased LH in bilaterally castrated 1-month-old bull. It may indicate that the pituitary gland of the 1-month-old bull is sensitive enough with the withdrawal of the negative feedback caused by the testicular steroids. Moreover, the present result is closer with the report that the aromatase was strongly expressed in the spermatids of the KE mice cryptorchid testes than of the

control testes (23). In the mouse, Sertoli cells were immunopositive only in the control testes, not in the cryptorchid testes (23). It was interesting that the present result indicated the localization of P450arom in the Sertoli cells of the cryptorchid testes, but not in the control testes. The heat-related-shift in the cellular localization of the aromatase enzyme can be speculated under prolonged exposure with increased heat.

In conclusion, prolonged exposure of the bovine testes with body heat may cause the downregulation of P450scc enzyme resulting reduced capacity for testosterone synthesis and no response of hCG treatment on testosterone secretion. Moreover, cryptorchidism may cause increased aromatase activity in the Sertoli cell that also increases with age, which may indicate the functional alteration of bovine testis exposed continuously to increased heat.

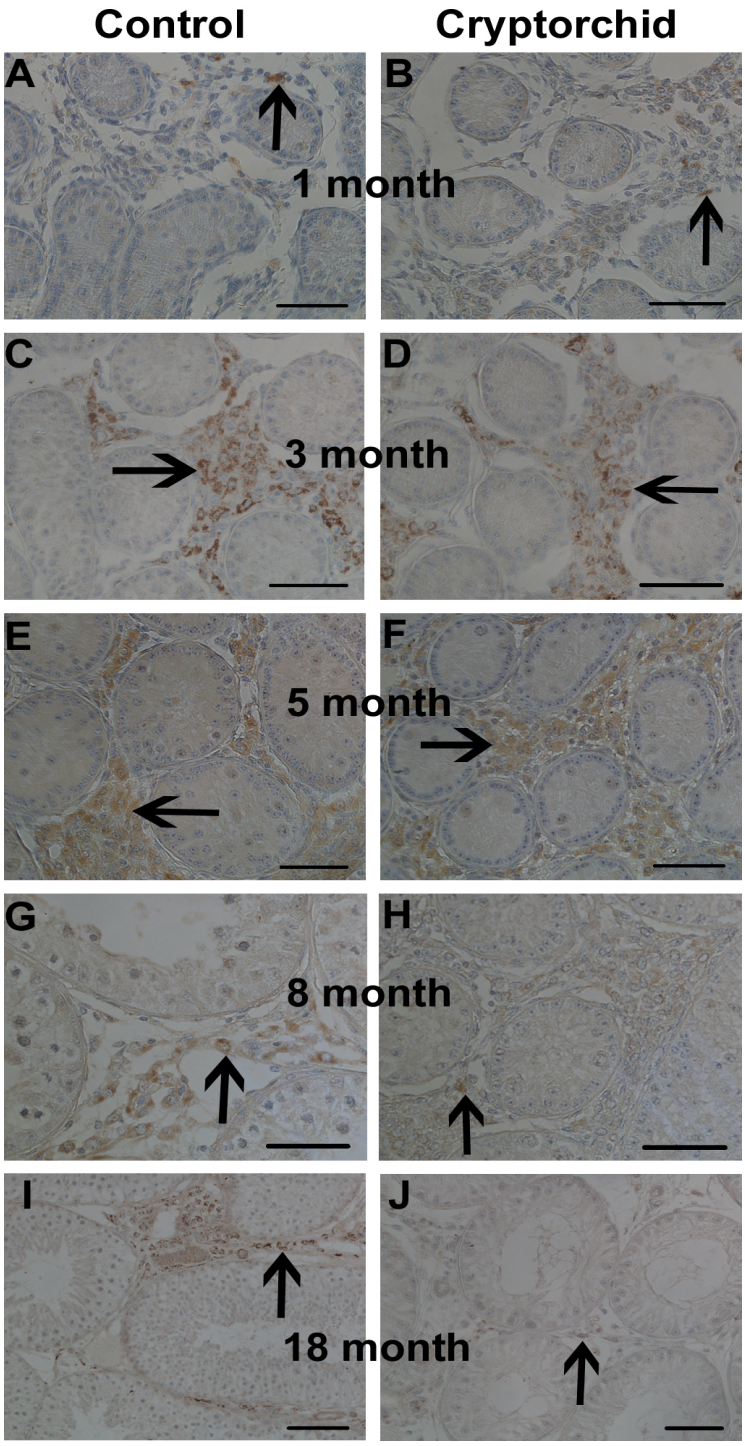


Fig. 5.B.1. Expression patterns of cytochrome P450scc enzyme in the Leydig cells (arrows) of paired testes from postnatally developing unilateral cryptorchid bulls. Expression is decreased in the 8-month-old and 18-month-old cryptorchid testes as compared to the contralateral scrotal control testes. Bars: 50 μm.

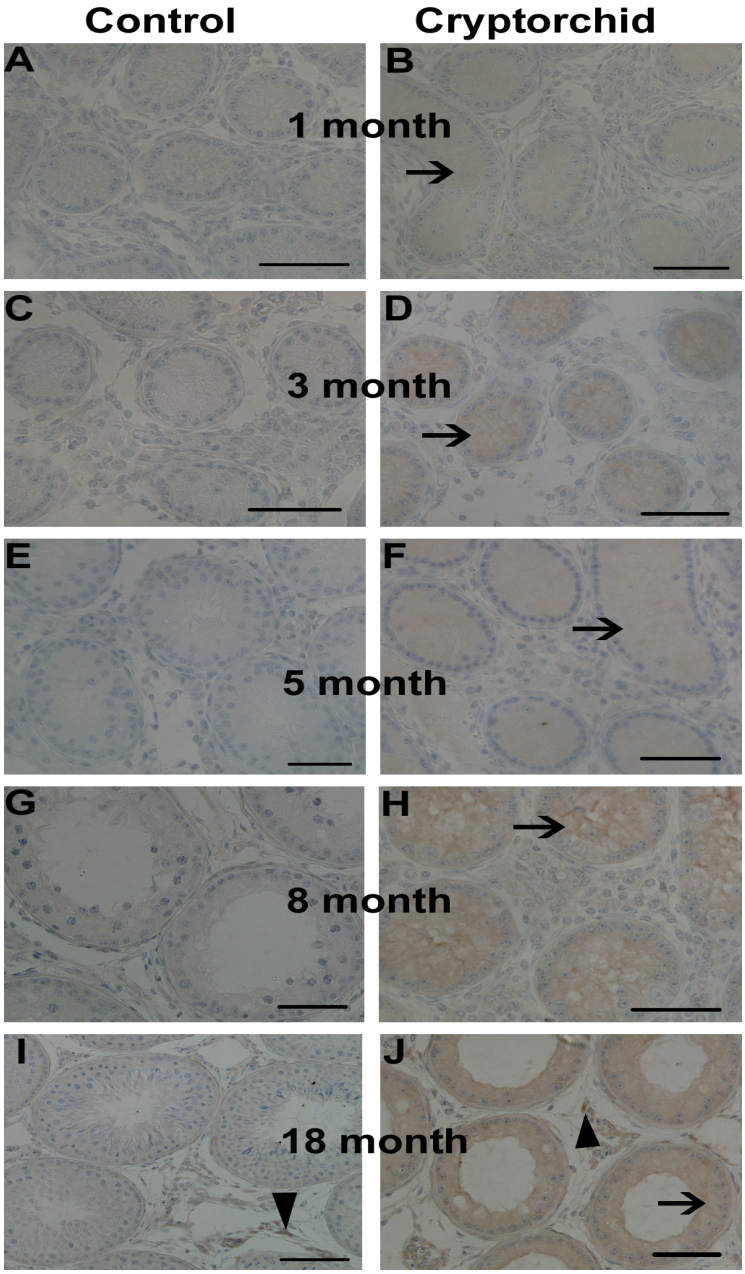


Fig. 5.B.2. Expression patterns of cytochrome P450arom enzyme in the Sertoli cells (arrows) and Leydig cells (arrow heads) of the paired testes from postnatally developing unilateral cryptorchid bulls. P450arom expression in the Sertoli cells is only present in the cryptorchid testis, which is increased as the animal matured. Leydig cells expressed P450arom in both the cryptorchid and scrotal testes, but only in 18 month-old bull, which is almost similar in both testes. Bars: 50 μm.

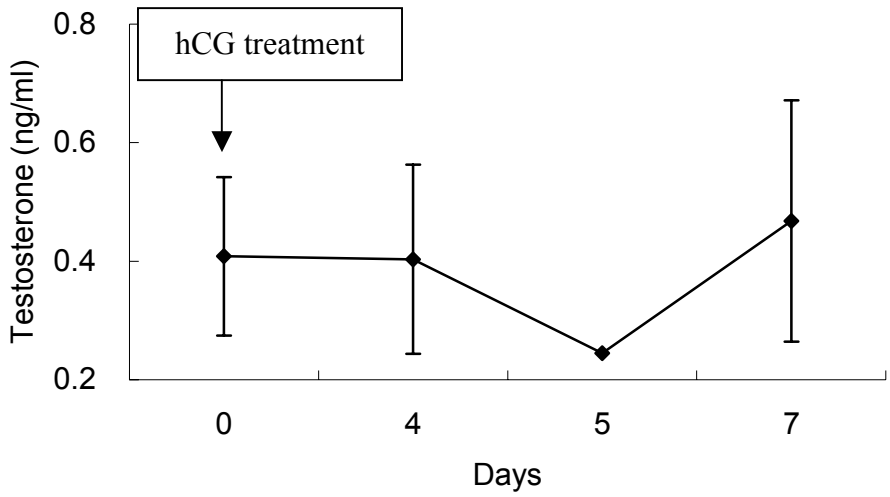


Fig. 5.B.3. Response of hCG treatment (2000-5000 iu IM) on testosterone concentration by the intact cryptorchid testis in bulls (n=3) after castrating the contralateral scrotal testis.

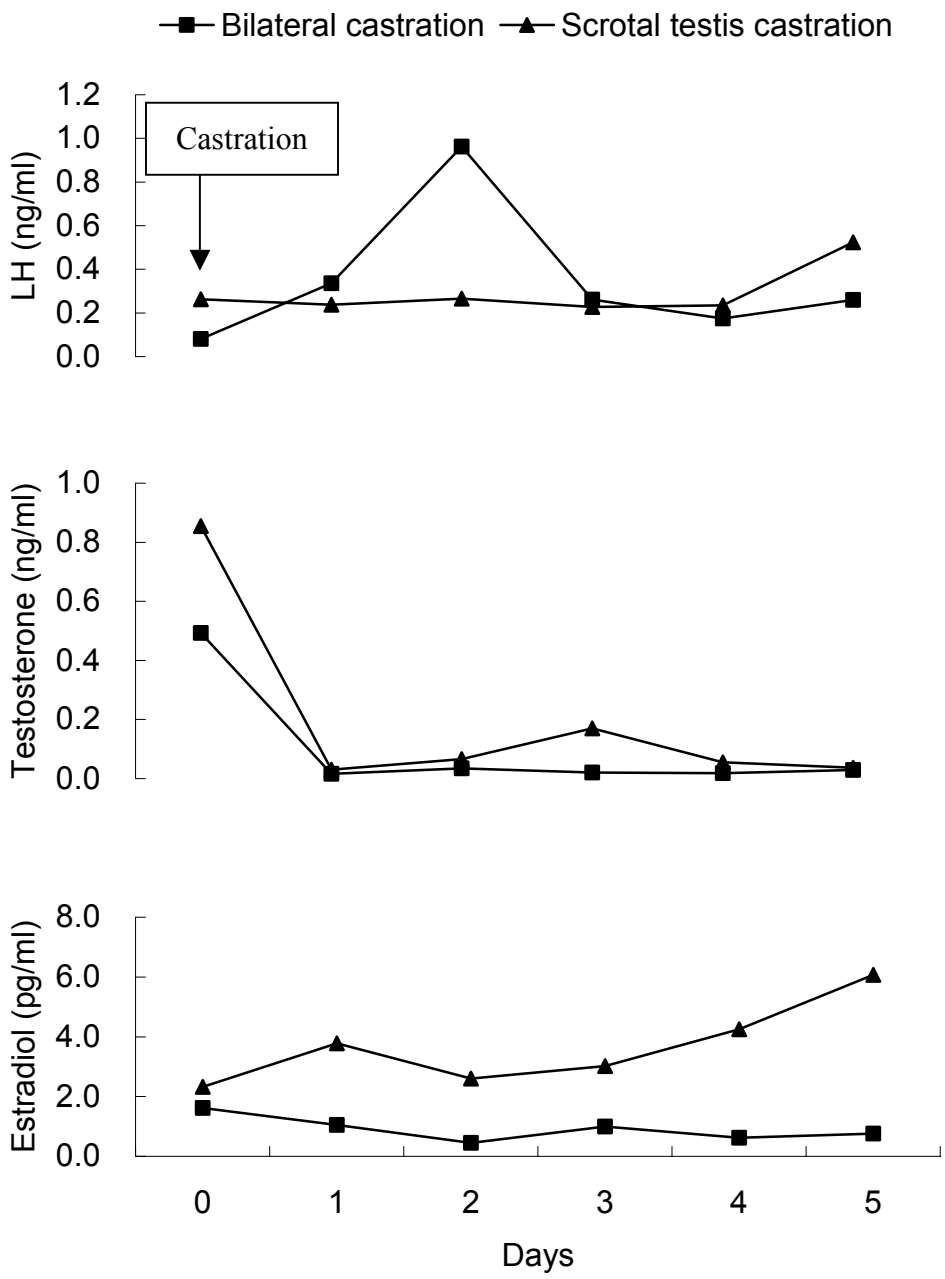


Fig. 5.B.4. Comparison of peripheral LH, testosterone and estradiol (E_2) concentrations in 1-month-old unilateral cryptorchid bulls for 5 days after bilateral castration of one bull and unilateral castration of scrotal testis leaving cryptorchid testis intact in another bull. Testosterone concentration is decreased in both bulls, while E_2 concentration is higher in scrotal testis castrated bull.

Chapter 5.C

Complex cystic lesion of rete testis of the abdominal testis and severe hypoplasia of the contralateral inguinal testis in the testosterone deficient, bilateral cryptorchid bull

5.C.1. Introduction

Cryptorchidism is a common congenital abnormality in which one or both testes fail to descend into the scrotum due to complex of genetic, hormonal, structural or other abnormalities. Descent of testis into the scrotum is possible only if the transabdominal descent followed by the inguinoscrotal descent is completed. In many species, it is reported that the transabdominal descent is androgen independent whereas the inguinoscrotal descent depends on androgen action (86). However, it was reported in the androgen-deprived male piglets (113) and in male rat pups (61) that testes were retained in the abdominal cavity. Cryptorchidism is relatively rare in bovine and the factors regulating testicular descent is not fully understood in this species.

Cryptorchidism is considered as an indicator of testicular dysgenesis, and it is often associated with the complications such as testicular neoplasia (92) or tubular atrophy and cystic hypoplasia of the rete testis (127). One of the very rare types of cystic lesion of the rete testis in human is reported to be the cystic dysplasia of rete testis (CDT). It is a benign, congenital lesion of the rete testis characterized by multiple, irregular, cystic spaces in the mediastinum of the testis that may involve the whole gonad. Only four cases out of thirty-six cases of human CDT reported until now are found in the cryptorchid testis (36, 126, 172, 173). It indicates a rare association of CDT and cryptorchidism, but may share common risk factors if occur together.

It was reported that the inhibition of cytochrome P450scc enzyme; a primary enzyme of the steroidogenic series essential in transforming cholesterol into pregnenolone, by toxicological or any other means causes the decreased production of testosterone in rats (17, 42, 117, 174). Suppression of androgen action or its production during neonatal life in rats induced the distension and over growth of rete testis, and severely reduced the testicular weight (112, 150). Moreover, testicular hypoplasia was also reported in a Chianina bull with XXY constitution of chromosomes (116). It was previously reported that the hypoplasia of the bovine cryptorchid testis in which the seminiferous tubules were immature, did not express peritubular α-smooth muscle actin (SMA); a marker specific to the differentiated myoid cells, until 8 months of age (53). However, the peritubular α-SMA in normally developing bovine seminiferous tubules appears at around 4 months of age (54). The present case report is the first case of its nature in bovine that demonstrated the complex cystic lesion of the abdominal testis and severe hypoplasia of the contralateral inguinal testis in 8-month-old bilaterally cryptorchid bull. In this bull, the postnatal changes of luteinizing hormone (LH) and testosterone concentrations were measured, immunohistochemically examined the localization of α-SMA and cytochrome P450 side chain cleavage (P450scc) enzyme in the testes sections, and did cytogenetic analysis with the objective to investigate the association between hormonal, cytogenetic and testicular abnormalities. Moreover, the comparison of disorders with other unilateral cryptorchid bulls was carried out.

5.C.2. Materials and Methods

5.C.2.1. History of animals

One bilateral cryptorchid (BC) and two unilateral cryptorchid (UC1 and UC2)

bulls, all Holstein-Frisian, were received at 5 weeks of age and reared together in the Obihiro University of Agriculture and Veterinary Medicine, Japan. Bull calves were fed with skim milk replacer (0-6 week), calf starter (6-12 week) and 18% calf grower (12-31 week) with grass hay and fresh water ad-libitum. The BC bull had an empty scrotal sac and a normal preputial orifice. On palpation, it had a very small testis like structure in the right inguinal canal that did not grow in size as the animal grew up normally. Both UC1 and UC2 bulls had one normally growing testis in the scrotum and another palpable cryptorchid testis in the left inguinal canal. The physical growth of all three bulls was similar. However, BC bull did not develop mounting behavior and was physically undermasculinized resembling to the female. The UC1 bull was bilaterally castrated at 20 weeks of age and later slaughtered at 23 weeks of age. Testes from BC and UC2 bulls were collected immediately after slaughter at 31 weeks of age. From the BC bull, a morphologically unidentifiable cystic mass from the abdominal cavity and a very small testis from the right inguinal canal were removed. The abdominal mass was expected to be a testicular anomaly. The gross findings of testes and postnatal changes of LH and testosterone concentrations were compared between three bulls, whereas the histological and immunohistological abnormalities of the testes sections were compared between the similar aged bulls, BC and UC2.

5.C.2.2. Histological and immunohistochemical studies

All testes samples and several tissue samples from the various parts of abdominal cystic mass of BC bull were fixed in Bouin's solution. The remaining of the cystic mass was preserved in phosphate-buffered 10% formalin for further study. Bouin fixed tissue samples were prepared for the histological and immunohistochemical

studies of α-SMA and P450scc enzyme as described in Chapter 2.3-2.5. The primary antibodies used were mouse monoclonal anti-α-smooth muscle actin (1:5000, Sigma Chemical Co., St. Louis, USA) and cytochrome P450 side chain cleavage polyclonal antibody in rabbit (1:200, CHEMICON International, Inc., CA 92590, USA). Instead of α-SMA and cytochrome P450scc primary antisera, the control sections were treated with normal horse serum and normal goat serum, respectively. Finally, the sections were counter-stained with Mayer's hematoxylin solution.

5.C.2.3. Hormone assay and cytogenetic analysis

Peripheral blood samples were collected weekly from 6 weeks of age until slaughter and plasma was harvested at –30^{o}C until assayed for LH and testosterone as described in chapter 2.6. Peripheral blood sample at 30 weeks of age from BC bull was also used for cytogenetic analysis. Chromosomal preparations were made by usual manner as described in chapter 2.7, and twenty metaphases were examined under high-resolution lens.

5.C.3. Results

On cytogenetic study, no chromosomal abnormalities were noticed, and normal 60, XY metaphases were observed in the peripheral lymphocyte culture.

5.C.3.1. Gross findings of the testes

The anatomical location of testes and their measurements in three bulls is shown in Table 5.C.1. Interestingly, the abdominal cystic mass (260 g, 18x 14x 5 cm^3) from BC bull (Fig. 5.C.1A) demonstrated multiple, anastomosing and irregular cystic spaces of

various size spreading from inside (Fig. 5.C.1B). Further examination of the formalin preserved abdominal cystic mass revealed the compressed rims of soft tissue while cutting the fibrous cystic walls (Fig. 5.C.1C). Surprisingly, the inguinal testis of BC bull was extremely hypoplastic (Fig. 5.C.1G) when compared with the inguinal testis of the similar aged UC2 bull and the younger UC1 bull that were also hypoplastic when compared with the scrotal testis of the same bull (Table 5.C.1).

5.C.3.2. Histopathological and immunohistochemical findings

Samples taken from the various parts of abdominal cystic mass of the BC bull demonstrated a few seminiferous tubules surrounded by the rete testis with flattened cuboidal epithelium, excessive connective tissue and blood vessels (Fig. 5.C.1D, E). The seminiferous tubules in the abdominal testis (Fig. 5.C.1F) as well as in the severely hypoplastic inguinal testis (Fig. 5.C.1H) of this bull were highly immature when compared with the seminiferous tubules of the UC2 bull, which were normal in the scrotal testis and relatively immature in the cryptorchid testis (Fig. 5.C.1F, H: left bottom). Moreover, several abnormal seminiferous tubules with swelling and intraluminal looping features were commonly observed in both testes of BC bull (Fig. 5.C.1F, H, I). However, the connective tissue and blood vessels were scarce in the severely hypoplastic testis (Fig. 5.C.1H, I).

Immunohistochemical staining revealed a similar pattern of absence of seminiferous peritubular α-SMA expression in both testes of BC bull (Fig. 5.C.2A) and in the cryptorchid testis of UC2 bull as well (Fig. 5.C.2B) while a normal pattern was observed in the scrotal testis of UC2 bull (Fig. 5.C.2C). On the other hand, the cytochrome P450scc enzyme was absent in the Leydig cells of both testes of BC bull

(Fig. 5.C.2D), but it was localized in both testes of UC2 bull (Fig. 5.C.2E, F), although its intensity was weak in the cryptorchid testis as compared to the scrotal testis. This result indicated that BC bull testes were deficient in cytochrome P450scc enzyme.

5.C.3.3. Postnatal changes of LH and testosterone concentrations

The postnatal changes of LH and testosterone concentrations in all three bulls are shown in Fig. 5.C.3A and B, respectively. Although not considerable, BC bull showed a low magnitude of transient rise in the LH level around 8 weeks of age, the following LH level until 19 weeks of age in this bull was lower as compared to that of UC1 and UC2 bulls (Fig. 5.C.3A). The most striking finding was the almost nadir testosterone level in the BC bull, which was similar as in UC1 bull after bilateral castration, while both UC1 and UC2 bulls showed the normal pattern of postnatal testosterone rise (Fig. 5.C.3B). This result, together with the absence of cytochrome P450scc enzyme activity in testes indicated the testosterone deficiency condition during postnatal development in BC bull.

5.C.4. Discussion

The gross and histological structures of the scrotal and cryptorchid testes of UC1 and UC2 bulls were consistent with the normal and cryptorchid testis structures for the respective ages, as reported previously (53, 54). Similarly, the postnatal changes of LH and testosterone concentrations in UC1 and UC2 bulls were almost similar to that of the normally growing bulls (9). Cryptorchid testes are generally hypoplastic, but the degree of hypoplasia observed in the inguinal testis of BC bull was very severe for its age. Interestingly, the gross and histopathological lesions of the abdominal testis of BC bull

resembled very much with the lesions of human cases of CDT (34, 36, 55, 126, 172, 173, 182). A similar case in human with a complex multilocular cystic transformation of the rete testis was reported having an association with smooth muscle proliferation, mimicking intratesticular Leydig cell neoplasm (64). Based on the absence of abnormal α-SMA expression pattern and other abnormally proliferating cells, such association with the neoplastic condition was not observed in the present case study. Moreover, any tissues foreign to the testis were not observed, despite of its gross similarities with the testicular teratoma reported in the cat (115). It can be suggested that the abdominal testis of BC bull was a case of complex cystic lesion of the rete testis, possibly a CDT of the bovine testis. Some reported cases of CDT in human have shown its frequent association with epsilateral renal anomalies (34, 36, 55, 101, 182). Such association was not found in the present case study. This is the first case study, which demonstrated in the same animal the association of postnatal testosterone deficiency and the incidence of bilateral cryptorchidism with the complex cystic lesions of one testis and severe hypoplasia of contralateral testis. The undermasculinization condition further supports the deprivation of androgen in BC bull, since androgens are essential for virilization of the male urogenital tract during embryogenesis and masculinization towards the male phenotype at puberty (33, 99).

The present data clearly demonstrated the absence of cytochrome P450scc localization in the Leydig cells of both testes of the BC bull, although its reason was not known. This condition might be responsible to cause the nadir postnatal testosterone level in this bull, similar as in the bilaterally castrated UC1 bull after 20 weeks of age. Since the condition of androgen deficiency or the loss of androgen receptors during neonatal life in rats resulted in the significant distension and over growth of rete testis,

reduced epithelial cell height and severe testicular hypoplasia (112, 150), it can be speculated that the prolonged postnatal testosterone deficiency in BC bull was responsible for almost similar, but more severe spontaneous conditions of the cystic lesion of rete testis with cuboidal epithelia in the abdominal testis and hypoplasia of the contralateral inguinal testis. It may be possible that the abdominal testis had enough space for the cystic enlargement, whereas the inguinal testis having limited space underwent hypoplastic shrinkage. Obstructive or secretory mechanisms were proposed as a possible cause of the isolated CDT in human (36), although its detail pathogenicity and relation to compromised androgenic action were not reported. Androgenic hormones regulate the epithelial and sub-epithelial physiology of the rete testis and other duct systems. It can be suggested that the testosterone deficiency may cause the functional alteration of the rete testis epithelia leading to the possible duct obstruction, derangements and cyst formation. Moreover, such alterations may become more severe if the normal contractile function of the rete testis is restricted by increased amount of non-contractile connective tissue, as observed in the cystic abdominal testis of BC bull.

Highly immature type of seminiferous tubules, some of them with abnormal swelling and intraluminal looping features, is another common histopathological finding of the BC bull testes. It may be related to the abnormal dysgenic development under extreme compression caused by the cyst walls in abdominal testis or by the severely reduced volume of the hypoplastic inguinal testis. Its detail pathogenicity needs to be further investigated. In conclusion, this is the first case reported in bovine, which is similar to human cases of CDT. These data further indicate the association between testosterone deficiency and the incidence of bilateral cryptorchidism as well as the occurrence of complex cystic lesion of the rete testis or the severe testicular hypoplasia.

Table 5.C.1. Anatomical location of testes and their measurements in one bilateral cryptorchid (BC) and two unilateral cryptorchid (UC1 and UC2) bulls.

Bulls (*)	Testes (**)	Side	Location	Weight (g)	Measurements (cm)		
					L	B	W
UC1 (20)	Scrotal (N)	Rt	Scrotum	32	5.5	3.5	2.5
	Cryptorchid (H)	Lt	Inguinal canal	18	4.2	2.5	2.0
UC2 (31)	Scrotal (N)	Rt	Scrotum	115	9.0	5.0	3.5
	Cryptorchid (H)	Lt	Inguinal canal	30	5.5	3.2	2.4
BC (31)	Cryptorchid (SH)	Rt	Inguinal canal	3	2.5	1.5	1.0
	Cryptorchid (C)	Lt	Abdomen	260	18.0	5.0	14.0

*Age (weeks) at testes removal, **testicular gross findings; N: normal, H: hypoplastic, SH: severely hypoplastic and C: cystic mass, Rt: right and Lt: left, L: length, B: breadth and W: width.

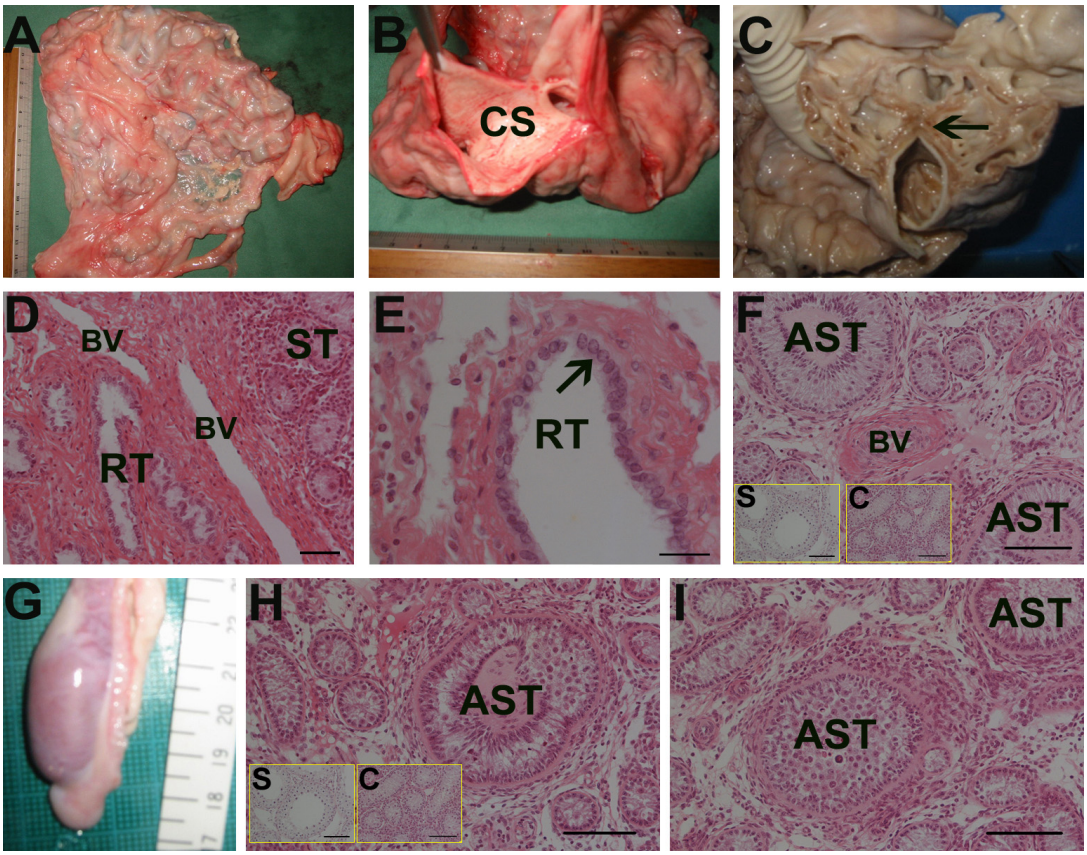


Fig. 5.C.1. The abdominal mass immediately after removal from a bilateral cryptorchid (BC) bull (A), after dissection of fresh sample showing multiple cystic spaces (CS) (B) and after preservation and dissection showing the compressed rims of soft tissue (arrow) in the cyst wall (C). The hematoxylin eosin staining of sections from the cyst wall (D, E, F) demonstrated a few seminiferous tubules (ST) surrounded by blood vessels (BV), excessive connective tissue (CT) and the rete testis (RT) with cuboidal epithelium (arrow). These sections revealed several abnormal seminiferous tubules (AST) with swelling or intraluminal looping features, and other highly immature seminiferous tubules, when compared with the seminiferous tubules of the scrotal (S) and cryptorchid (C) testis sections of the similar aged UC2 bull (left bottom, F). The severely hypoplastic, contralateral inguinal testis of BC bull (G). On hematoxylin eosin staining of the sections (H, I), this testis also demonstrated highly immature seminiferous tubules (as compared to the similar aged UC2 bull, in the left bottom, H) and several AST, but the CT and BV were scarce. Bars: D: 50 µm; E: 25 µm; F, H and I: 100µm.

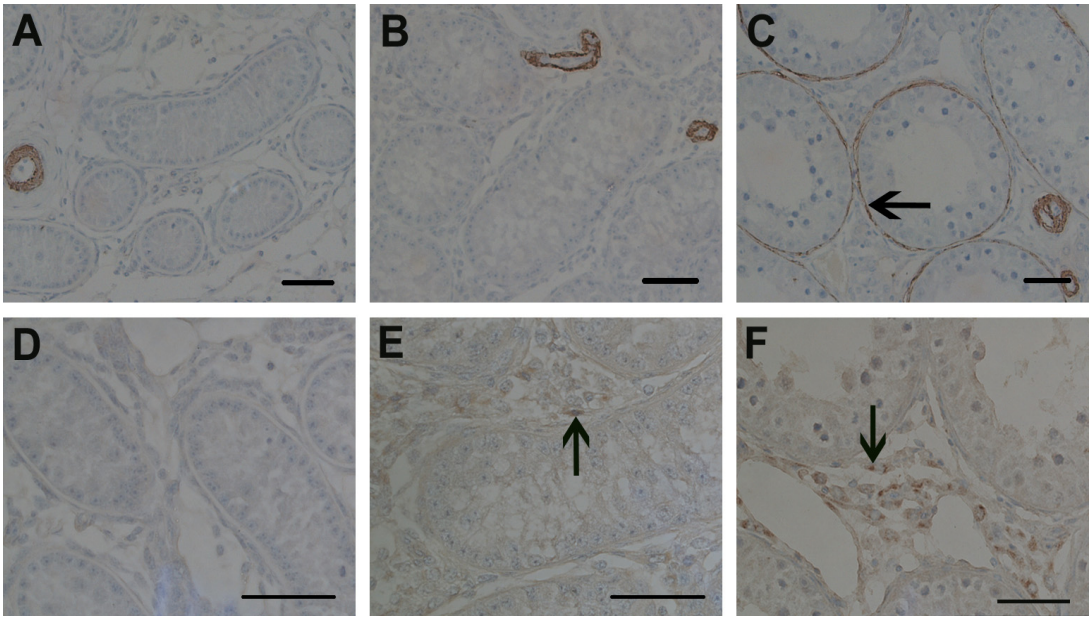
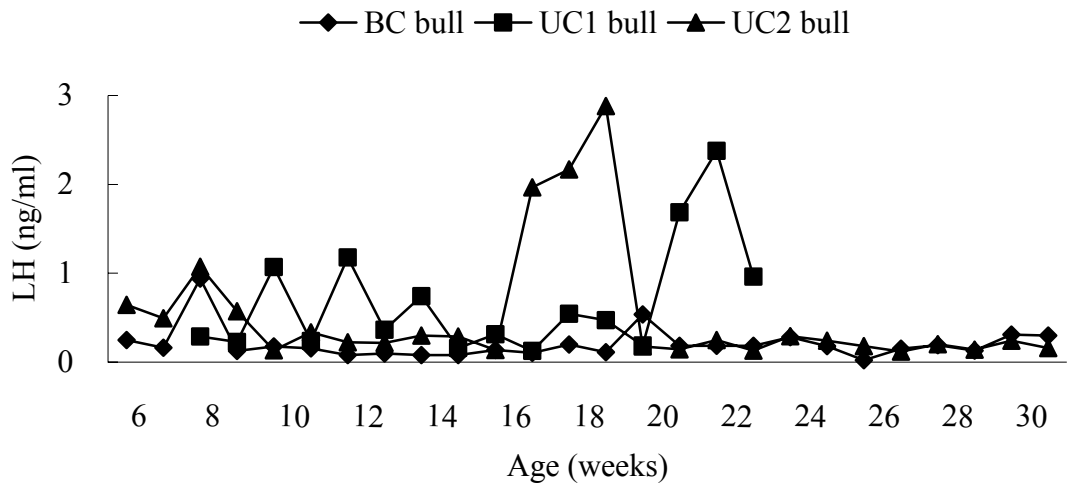


Fig. 5.C.2. Immunohistochemical localization of α-SMA and cytochrome P450scc enzyme in the testis sections of a bilateral cryptorchid (BC) and a unilateral cryptorchid (UC2) bull. The seminiferous peritubular α-SMA was absent in both testes of BC bull (A, abdominal testis section) as well as in the cryptorchid testis of UC2 bull (B), but it showed a normal regular pattern (arrow) in the scrotal testis of UC2 bull (C). The cytochrome P450scc enzyme was absent in the Leydig cells of both testes of BC bull (D, inguinal testis section). This enzyme was localized in the Leydig cells (arrow) of both testes of UC2 bull (E, cryptorchid testis section, F, scrotal testis section), but its intensity was weak in the cryptorchid testis (E) as compared to the scrotal testis (F). Bars: 50μm.

A.



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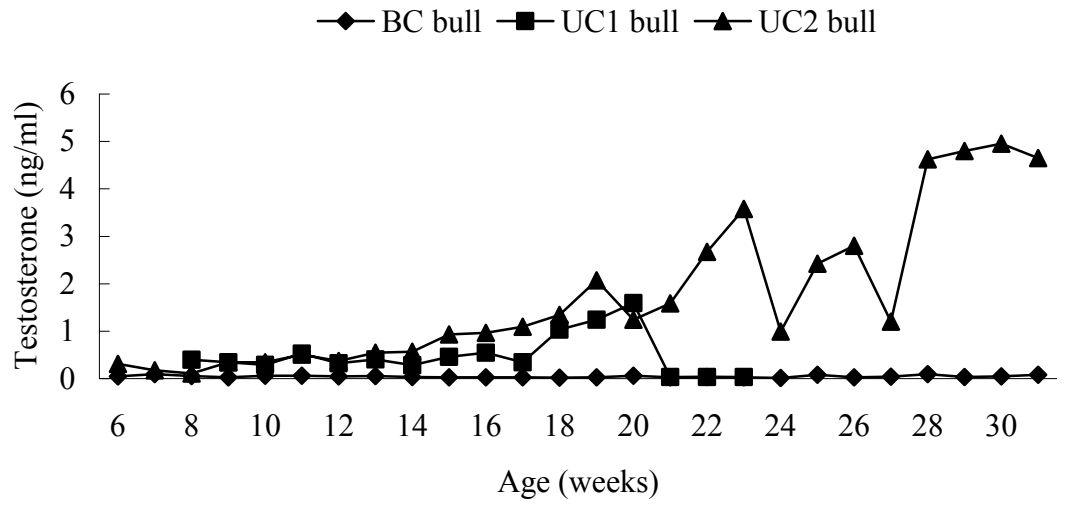

Fig. 5.C.3. Postnatal changes of LH (A) and testosterone (B) concentrations in one bilateral cryptorchid (BC) and two unilateral cryptorchid (UC1 and UC2) bulls. UC1 was bilaterally castrated at 20 weeks of age and slaughtered at 23 weeks of age. UC2 and BC were slaughtered at 31 weeks of age. Weekly blood samples were collected from 6 weeks of age until slaughter. Note the almost nadir testosterone level until 31 weeks in BC bull, similar as in the UC1 bull after bilateral castration.

Chapter 5.D

Pathogenesis of the abnormal swelling and intraluminal looping of the seminiferous tubules in the cystic dysplastic or severely hypoplastic bovine testis

5.D.1. Introduction

Cryptorchid testes generally reveal different types of morphological and cellular abnormalities. Previously in chapter 5.C, a case of complex cystic lesion of the abdominal testis and severe hypoplasia of the inguinal testis was described in the bilateral cryptorchid bull. Both testes of this bull demonstrated several abnormal seminiferous tubules (STs) with characteristic swelling and intraluminal looping features. Literature review did not reveal such kind of ST abnormality in the similar cases of cystic dysplastic human testis (36, 71, 126), and in the hypoplastic, but less severe case of the boar (141) and bull (53) cryptorchid testis. However, it was reported that a few abdominal cryptorchid testis in human comprised of the hypoplastic nodule of ST, which constituted extremely pleated annular STs characterized by the immature and dysgenic Sertoli cells (SCs) (147).

In the testis, SCs are the major epithelial cells of ST, which are separated with the peritubular cells by the basement membrane (BM). Vimentin specifically localizes in the SC perinuclei and may help to stabilize them basally in the bovine testis (54). It was demonstrated that co-cultures of SCs and peritubular cells develop complex morphological structures, which resemble the gross architecture of ST (176). Further studies have demonstrated that mono-cultured or co-cultured SCs and peritubular cells can secrete number of specific proteins required for the synthesis of BM (51, 166), and

both cell types express BM gene *in vitro* (149). The present study is a more specific investigation on ST anomaly of the previously reported case in chapter 5.C, which aims to describe the series of abnormal microstructural changes, analyze the altered pattern of vimentin expression, and explain the pathogenesis of ST swelling and intraluminal looping. In this study, it is also demonstrated that the aggregation of immature SCs, *in vivo*, may be able to synthesize the morphological structures that resemble to BM, and vimentin may play a key role to align the aggregated SC nuclei basally.

5.D.2. Materials and methods

Bouin fixed samples from both, the cystic abdominal and severely hypoplastic inguinal testes obtained as explained in Chapter 5.C.2.1 from the bilateral cryptorchid bull, were sectioned (5 μm), stained with hematoxylin-eosin (HE) stain and examined for the presence of abnormal ST. The tissue blocks producing such abnormal tubules were selected for serial sectioning. Several serial sections (3 μm) were prepared from such selected blocks, stained with HE stain and examined under light microscope fitted with a digital camera and a display monitor (MICROPHOT-FX, Nikon, Japan), and serial wise photographs of the same abnormal tubule were taken. These photo-series were studied to identify two ends of the abnormal ST, and the series of microstructural and cellular deviations were followed from one end. The other 5 μm sections were stained immunohistochemically against vimentin as described in chapter 2.5. The polyclonal anti-calf lens vimentin (1:150; MEDAC, Hamburg, Germany) was used as primary antibody, and the control sections were treated with normal goat serum instead of the primary antiserum.

5.D.3. Results

The important events of the ST alteration during abnormal swelling and intraluminal looping, which was observed serially, is shown in Fig. 5.D.1A-I. Interestingly, it was observed that two different parts of the same ST was undergoing abnormal swelling and intraluminal looping, which are denoted by tubule 1 and tubule 2 (Fig. 5.D.1A-I). Since both parts demonstrated a similar pattern of alteration, the description here follows only for tubule 1. The end point of a series of abnormal development demonstrated a portion of the collapsing ST, the aggregation of SC nuclei and isolation of the tubule (Fig. 5.D.1A, B). This was followed by a typical basal alignment of SC nuclei with a few squeezed SC nuclei and germ cells in the lumen, and gradual swelling of the tubule (Fig. 5.D.1C, D). Surprisingly then, another aggregation of SC nuclei gradually appeared in the central part of lumen of the swollen ST (Fig. 5.D.1E). This event was followed by gradual appearance of a new intraluminal morphological BM and the basal alignment of aggregated SC nuclei, but in a more compact fashion, onto the new BM in a concentric circle forming an intraluminal loop (Fig. 5.D.1F). This loop persisted in a few sections (Fig. 5.D.1G). This was further followed by the exact reverse order of events, such as the gradual loss of basal alignment of SC nuclei in the intraluminal loop and appearance of the aggregation of SC nuclei in the central part of swollen ST lumen (Fig. 5.D.1H), and reduction of the ST diameter (Fig. 5.D.1I) leading to the other collapsing end. The SCs of such tubules appeared dysgenic with round or elongated nuclei, characteristics of a highly immature type of SC (75, 163). All SCs in the swollen and intraluminal loop expressed the perinuclear vimentin, which was disrupted and carried by the squeezed SC nuclei as well (Fig. 5.D.2A-C). Interestingly, a swollen ST from 5 μm sections also demonstrated

an invaginating portion (Fig. 5.D.2A), which was not noticed in the serially prepared sections.

5.D.4. Discussions

It could be possible that the inward pressure from two opposite sides along the longitudinal axis of ST might have been exerted by the cyst walls in the cystic abdominal testis, and due to severely reduced volume in the hypoplastic inguinal testis. The invaginating portion of swollen ST (Fig. 5.D.2A) further supports for this pressure hypothesis. It can be speculated that the pressure might have caused the collapse of two opposite ends, swelling of the central portion of the tubule and disruption of SC vimentin making the SC nuclei squeeze possible. The most striking demonstration of this study is the possibility of forming, *in vivo*, the morphological structures that resemble with BM by the aggregating immature SC, which is highly consistent with the principles of previous demonstrations *in vitro* (51, 166, 176) or *in vivo* (51). It can be proposed that vimentin may play a crucial role during basal alignment of aggregated SC nuclei, as vimentin is vital for the basal positioning of SC nuclei when they move towards the lumen while undergoing maturation in the normally developing bovine testis (54). Alteration and disruption of the Sertoli cell vimentin was reported in rats (179) and rhesus monkeys (186) after experimental cryptorchidism, but not in the less severe, hypoplastic bovine cryptorchid testis (53). However, none of the conditions have reported the abnormal development of STs with swelling and intraluminal looping features. The present results suggest that, in a cystic dysplastic or severely hypoplastic testis, ST may undergo compression along the longitudinal axis from both sides resulting a characteristic tubular swelling and intraluminal looping towards the center of

pressure. The aggregation of immature SCs, *in vivo*, may be able to synthesize the morphological structures that resemble to BM, and vimentin may play a role to align SC nuclei basally.

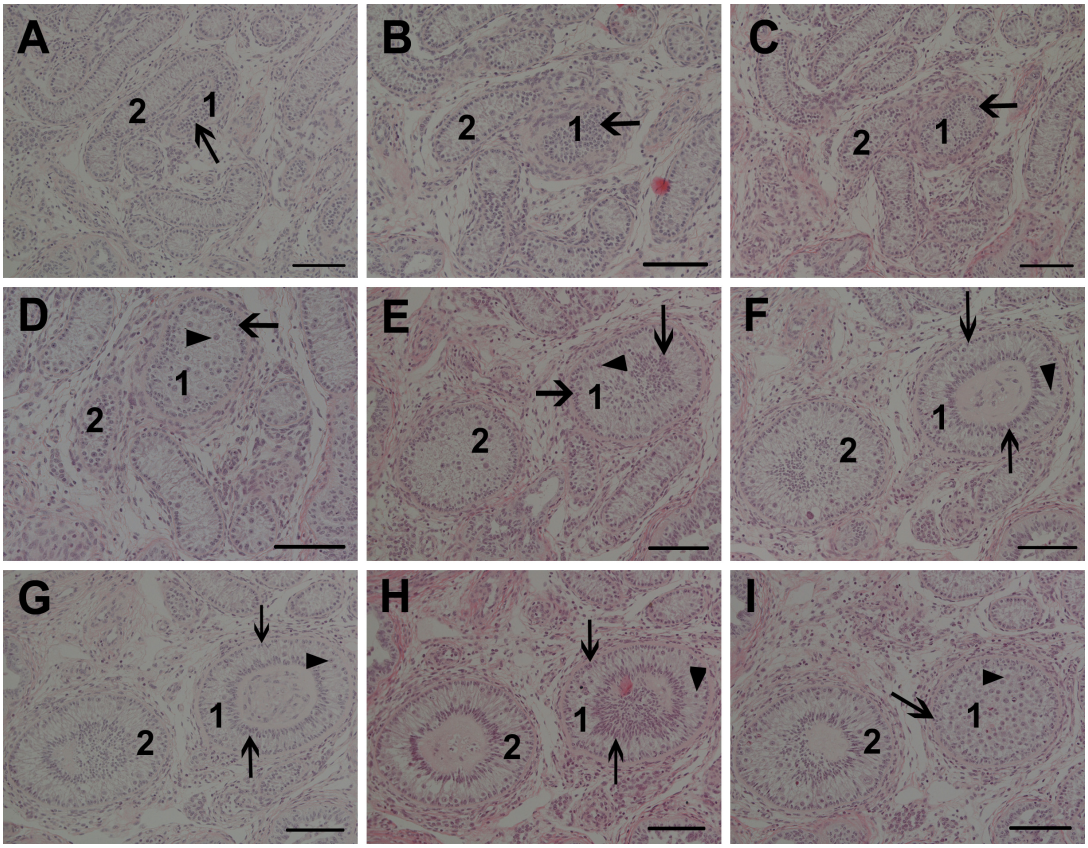

Figure 5.D.1. Important events of cellular and microstructural alterations in series, in a seminiferous tubule (ST) undergoing looping anomaly. Tubule 1 and tubule 2 denote for two different parts of the same ST. Both parts demonstrated a similar pattern of alterations and the description here follows only for tubule 1. All arrows indicate for Sertoli cell (SC) nuclei and arrowheads indicate for germ cells. Hematoxylin-eosin stained sections showing a portion of the collapsing ST, aggregation of SC nuclei and isolation of the tubule (A, B), the gradual swelling of tubule (C, D), appearance of another SC nuclei aggregate inside the swollen ST lumen (E), appearance of a new intraluminal basement membrane (BM) and basal alignment of the SC nuclei onto the new BM forming a loop (F), that persisted in a few sections (G). The reverse order of events characterized by the gradual loss of basal alignment of the SC nuclei in the intraluminal BM (H) and reduction of the swollen ST diameter (I). Bars: 100 μm.

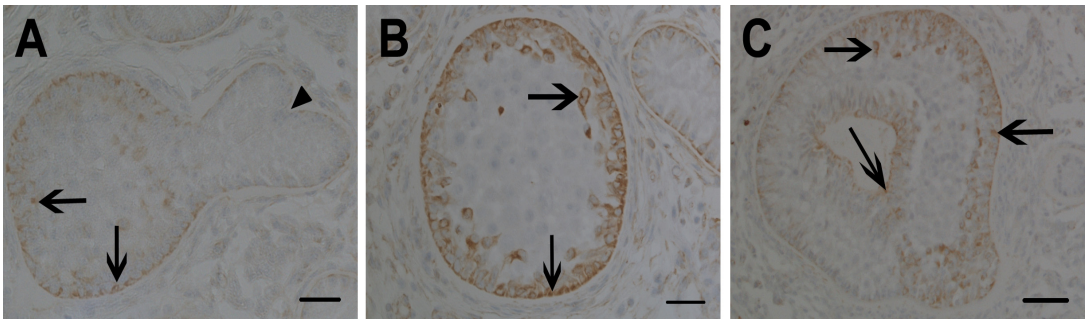

Figure 5.D.2. Vimentin immunoreactions of representative events of seminiferous tubule (ST) looping anomaly. All arrows indicate for Sertoli cell (SC) vimentin. All SC in the swollen (A, B) and looped (C) ST expressed a strong perinuclear vimentin, which was disrupted and carried by the squeezed SC nuclei. An invaginating portion (arrowhead) of a swollen ST (A). Bars: A, B: 25 μm, C: 50 μm.

Chapter 6

Cellular, Some Cytoskeletal and Functional Alterations in the Bovine Testis After One Week of Scrotal Insulation

Chapter 6.A

Alterations in the histological and immunohistochemical localization patterns of α-smooth muscle actin (SMA), vimentin and cytokeratin

6.A.1 Introduction

The scrotal skin contains numerous sweat glands, which are important to maintain the testicular temperature below the body temperature and to maintain the normal testicular functioning. If this scrotal thermoregulatory mechanism is interfered by any means, such as by scrotal insulation, spermatogenesis may be arrested. Scrotal insulation model has been used in the past to induce increased heat in the testis while studying the effect of heat on spermatogenesis. In bulls, the increased testicular temperature induced by scrotal insulation adversely affects spermatogenesis, which is reflected in the post insulation semen quality (32, 94, 178).

Testicular functioning is highly related to its cellular and structural integrity. Cytoskeletal proteins provide structural and functional support to cells. The expression patterns of α-smooth muscle actin (SMA) and vimentin in the bovine testis indicate the maturation status of seminiferous tubules (53, 54). The continuous exposure of the postnatally developing bovine testis to body heat causes failure of spermatogenesis and delayed appearance of α-SMA, and the Sertoli cell vimentin fail to attain the mature pattern (53). Apart from causing the degenerative lesions, induced cryptorchidism also causes the structural disruption of actin filaments in the rat testis 108). Similarly, alteration and disruption of the Sertoli cell vimentin in the rat (179) and rhesus monkey (186) testis have been reported after experimental cryptorchidism. The expression of cytokeratin in the adult seminiferous epithelium has been regarded as a sign of

regaining undifferentiated immature feature in the testis of rhesus monkey after experimental cryptorchidism (186). The present study investigates the effects on the spermatogenic cells and also on the immunohistochemical expression of α-SMA, vimentin and cytokeratin after increased testicular heat in bulls induced by scrotal insulation for 1 week.

6.A.2. Materials and methods

6.A.2.1. Scrotal insulation

Two bulls (12 and 14-month-old) were unilaterally castrated, and after 2-3 weeks, subjected for contralateral scrotal insulation for 1 week, then the castration of insulated testis. Normal pre-scrotal-insulation testes were used as control against the insulated testes. Scrotal insulation method for bull described elsewhere (Brito et al., 2003) was followed with minor modifications as shown in Fig. 6.A.1. In brief, after complete recovery from the first castration, the remaining second testis was prepared for insulation. Testis was covered with thin cotton layer, wrapped with a diaper, fixed lightly without pressure by applying a single layer of bandaging tape (3M Vetrap^{TM}, 3M Animal Care Products, St. Paul, MN, USA) and put into the cotton bag designed with open part having the purstring mannered cotton threads slipped into opposite direction, that secured the bag and the contents into the scrotal neck. The insulation was further fixed by gum-tape around the scrotal neck and maintained dry for 1 week. This experiment was performed at National Livestock Breeding Center (NLBC), Nikkappu station (Hokkaido, Japan) in the month of July 2004, where the Mean ± SEM of the maximum and minimum temperature and humidity of room where bulls were kept during insulation period was 27.9 ± 0.8^{o}C, 19.9 ± 1.0^{o}C and 79.6 ± 1.8%, respectively.

Similarly, the Mean ± SEM of the temperature between the skin and insulating material, and the rectal temperature in 2 bulls was 37.7 ± 0.3^{o}C, 36.5 ± 0.2^{o}C and 38.6 ± 0.2^{o}C, 38.9 ± 0.2^{o}C, respectively.

6.A.2.2. Tissue preparation and immunohistochemical staining

Testis samples were fixed in Bouin's solution, blocked in paraffin, and sectioned (5 μm) and stained specific immunohistochemical staining for α-SMA, vimentin and cytokeratin as described in chapter 2.3-2.5. The primary antibodies used were the mouse monoclonal anti-α-smooth muscle actin (1:5000; Clone No. 1A4, Sigma Chemical Co., St. Louis, USA), polyclonal anti-calf lens vimentin (1:150; MEDAC, Hamburg, Germany) and the rabbit polyclonal anti-keratin (1:100, DAKO, Copenhagen, Denmark). The control sections were treated with normal horse and normal goat serum instead of α-SMA and vimentin or cytokeratin primary antisera, respectively. Finally, the sections were counterstained with Mayer's hematoxylin solution.

6.A.3. Results

6.A.3.1. Histopathological lesions of the seminiferous tubules

Histopathological comparison between the control and insulated testis is demonstrated in Fig. 6.A.2.A-C. Control testes demonstrated normal seminiferous tubules with different stages of spermatogenesis (Fig. 6.A.2.A). On the other hand, scrotal insulation for one week caused the degenerative lesions of seminiferous tubules, characterized by a deformed peritubular structure and loss of Sertoli cell processes in the severely degenerated tubules (Fig. 6.A.2.B), loss of spermatogenic cells and presence of vacuoles, pyknotic nuclei, and multinucleated giant cells (Fig. 6.A.2.B, C)

in other tubules. Spermatogenic cell degeneration was of various types ranging from the total loss of spermatocytes or more advance cells to a loss of spermatids and spermatozoa (Fig. 6.A.2.B).

6.A.3.2. Alterations in the immunohistochemical expressions of α-SMA, vimentin and cytokeratin

As compared to the control testis (Fig. 6.A.3.A), seminiferous peritubular α–SMA in the insulated testis (Fig. 6.A.3.B) demonstrated a considerable distorted pattern in the severely degenerated tubules, while the less pronounced changes were observed in the tubules with relatively mild degeneration. Sertoli cell vimentin expression pattern is almost unaltered in the insulated testis (Fig. 6.A.4.B) when compared with the control testis (Fig. 6.A.4.A). Sertoli cells of the control and insulated testes (not shown) did not express cytokeratin.

6.A.4. Discussion

Pachytene spermatocytes and young spermatids were found to be the first cells affected after experimental cryptorchidism in rats (25, 51). When cultured the fragments of human testes at 37^{o}C, the number of spermatogonia and spermatocytes were not affected, whereas the spermatids and spermatozoa were lower (123). The present result indicated that spermatozoa and spermatids in the bovine testis are most susceptible with increased heat. Moreover, degeneration of spermatocytes, and changes in the Sertoli cells and peritubular structure post insulation may indicate towards prolonged recovery time even after the removal of thermal stress. It can be suggested that these changes may be directly related to the morphological abnormalities of ejaculated spermatozoa

and decreased sperm production post insulation (32, 94, 178).

It was suggested that the rat testis under local heat *in vivo* shows effects on the Sertoli cells and basement membrane of seminiferous tubules (87). Moreover, in association with the degeneration of the germ cells, induction of experimental cryptorchidism in rats affected the structure and function of Sertoli cells and myoid cells morphology (97). The present finding of a considerable distorted pattern of the peritubular α-SMA in the severely degenerated tubules than in the tubules with relatively mild degeneration may indicate the relationship of peritubular α–SMA with the degree of degenerative and structural changes of the seminiferous tubules post insulation. This finding is in agreement with the suggestion that actin filament arrangements in the myoid cells of testes are affected with thermal stress and also reflects the spermatogenic activity (107). It can be, therefore, suggested that either a sudden thermal stress that causes the degenerative changes of seminiferous tubules, or a diminished internal pressure of the seminiferous tubules due to depleted germ cells, might have caused a distorted pattern of the peritubular α-SMA in severely degenerated tubules post insulation. The α-SMA may be more sensitive to increased heat.

Impaired Sertoli cell function (73, 74) and altered distribution of Sertoli cell vimentin filaments have been reported in the rat testis (179) and rhesus monkey testis (186) after experimental cryptorchidism. The present data did not reveal any considerable change in the immunohistological expression pattern of vimentin in the bovine testes post insulation. Cytokeratin is expressed in the seminiferous tubules of spontaneous cryptorchid testis in human (153) and after experimental cryptorchidism in rhesus monkey (186), which is considered as a sign of undifferentiated immature feature of Sertoli cells. However, cytokeratin was not expressed in this study after 1 week of

scrotal insulation. The present finding may indicate that vimentin is not sensitive to heat and cytokeratin is not expressed in the bovine testis after 1 week of scrotal insulation. Further study may be required to study about the sensitivities of α-SMA, vimentin and cytokeratin in the bovine testis after prolonged period of heat treatment.

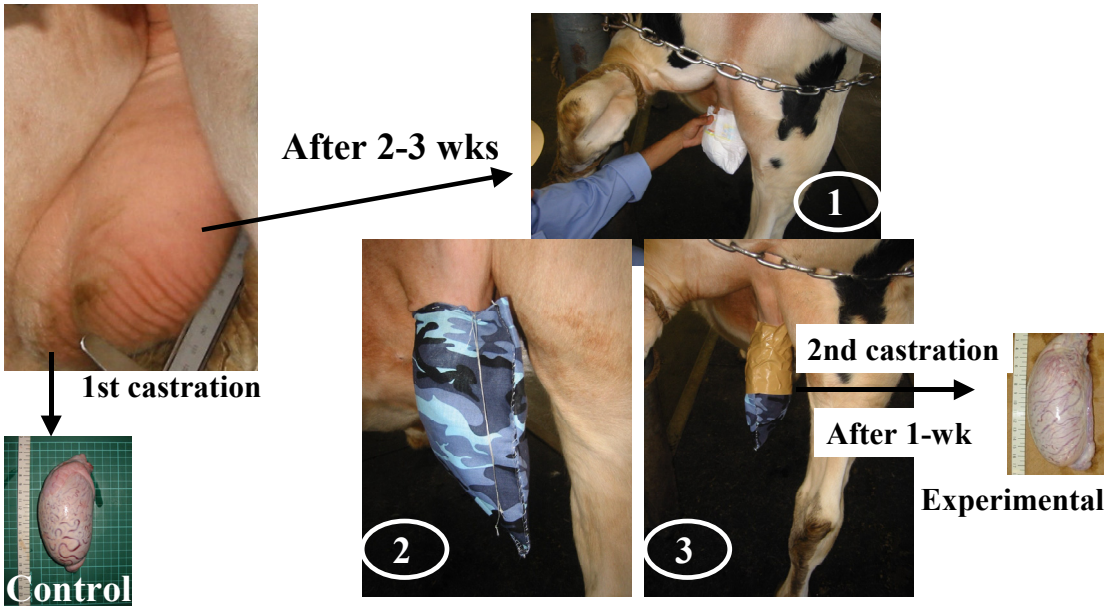

Fig. 6.A.1. Methodology of unilateral castration (first, control) followed after 2-3 weeks by contralateral scrotal insulation for 1 week and castration of the insulated (second, experimental) testes. The scrotum was surrounded by a think layer of cotton and a disposable diaper (1), held in place by a single layer of bandage and a cotton bag with a neck fastened in purstring manner (2) and supported by placing tape around the neck of the scrotum (3).

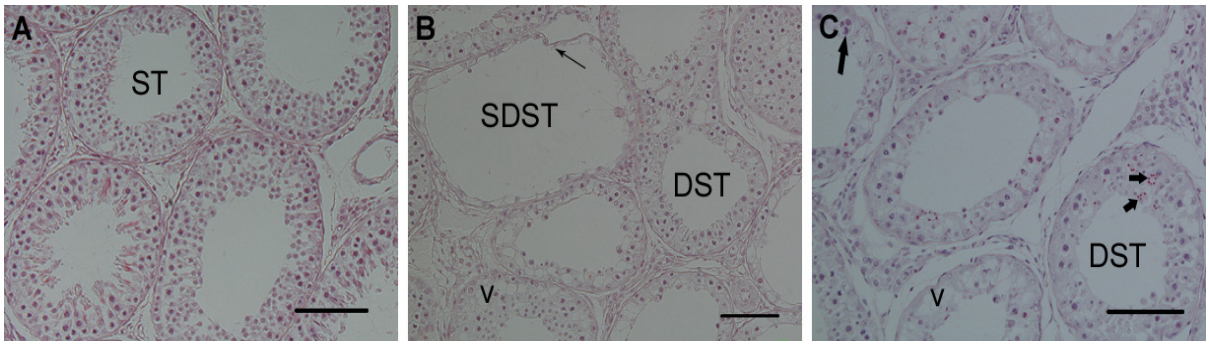

Fig. 6.A.2. Hematoxylin-eosin stained sections of control (A) and insulated (B, C) testes. Insulation caused lesions of spermatogenic cell degeneration and irregular outer structure of the seminiferous tubules that was more evident in severely affected tubules (thin arrow). ST: Normal seminiferous tubule, SDST: severely degenerated tubule with only the basal cell lining is intact, DST: degenerated tubule, v: vacuoles, Long thick arrow: multinucleated giant cells, short thick arrows: pyknotic nuclei. Bars: 100 μm.

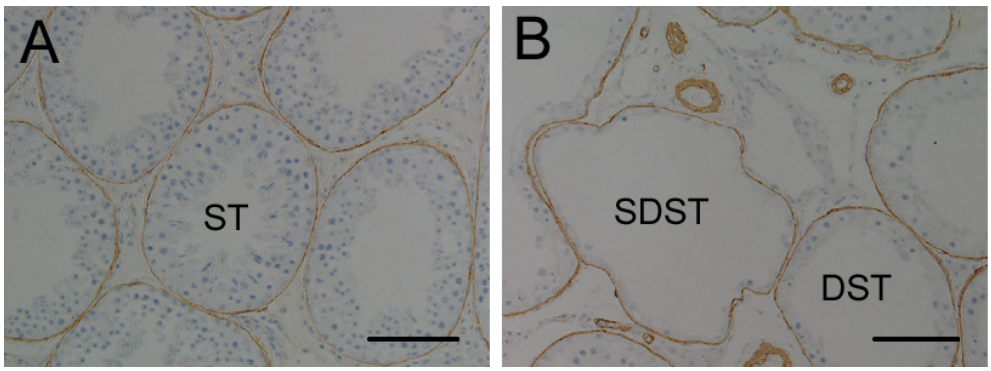

Fig. 6.A.3. Immunohistochemical expression patterns of α-smooth muscle actin (SMA) in the control (A) and scrotal insulated (B) testis. ST: normal seminiferous tubule, SDST: tubule with severe spermatogenic degeneration, DST: tubule with relatively mild spermatogenic degeneration. Bars: 100 μm.

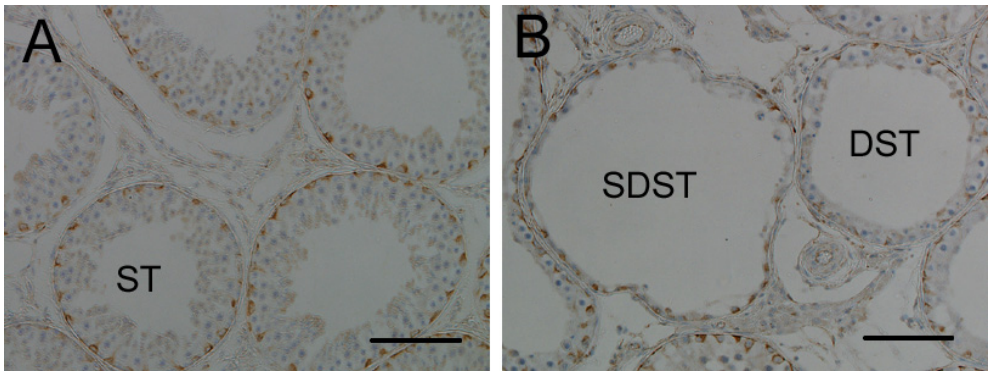

Fig. 6.A.4. Immunohistochemical expression patterns of vimentin in the control (A) and scrotal insulated (B) testis. ST: normal seminiferous tubule, SDST: tubule with severe spermatogenic degeneration, DST: tubule with relatively mild spermatogenic degeneration. Bars: 100 μm.

Chapter 6.B

Alterations of the steroidogenic functions

6.B.1. Introduction

Bovine testes in the scrotum are maintained at about 5^{o}C below body temperature (95). The scrotal insulation model is useful to induce increased testicular temperature, which results in decreased semen quality post insulation (32, 94, 178). Moreover, induced testicular degeneration by scrotal insulation in bulls (144, 161) and due to pathological testicular degeneration in rams (26, 27) has been shown to cause decreased peripheral testosterone level. Similarly, increased testicular temperature due to cryptorchidism or local heating of the rat testis has been shown to reduce the testosterone production (49, 68). However, the reason of this reduced steroidogenesis by the testis under thermal stress is not clear, and the possible effect on the enzymes involved in steroid biosynthesis need to be further investigated. The downregulation of cytochrome P450 side chain cleavage (P450scc), an enzyme responsible for the conversion of cholesterol into pregnenolone in the testosterone synthesis pathway, in the bovine cryptorchid testis after 8 months of age, was observed in Chapter 5.B, which also indicated almost no response to hCG treatment on testosterone production by the cryptorchid testis. Therefore, the present study was designed to investigate the hormonal changes in bulls under testicular thermal stress, and to establish whether the downregulation of P450scc enzyme was caused by increased heat in the testis. The present study model is the first of its kind, which used one testis for control and another contralateral testis from the same animal for inducing thermal stress.

6.B.2. Materials and methods

The present experiment was performed at National Livestock Breeding Center (NLBC), Nikkappu station (Hokkaido, Japan) in July 2004. Two bulls (Bull A, 12-month-old and Bull B, 14-month-old) were unilaterally castrated (first castration), and after 2-3 weeks, subjected for contralateral scrotal insulation for 1 week (from July 21 to July 28) then the castration of insulated testis (second castration), as shown in Fig. 6.A.1. Scrotal insulation method for bulls described elsewhere (Brito et al., 2003) was followed with minor modifications. Daily scrotal temperature was measured by inserting a thermometer between the insulating material and the scrotal skin. Similarly, daily rectal temperature of the animals and the environmental conditions such as the room temperature and the humidity during insulation period was also recorded.

Peripheral blood samples were collected once a week from 1 week before (Bull A), or from the day of (Bull B), first castration until 3 weeks after second castration, and daily during 1 week of insulation period. The concentrations of LH, testosterone and estradiol were measured as described in Chapter 2.6. Moreover, normal pre-scrotal-insulation testes were used as control against the contralateral insulated testis. Immunohistochemical staining was performed for P450scc to compare the expression patterns between the control and insulated testes. The primary antibody used was the polyclonal antibody in rabbit, cytochrome P450 side chain cleavage (1:200, CHEMICON International, Inc., CA 92590, USA). The control sections were treated with normal sera instead of the primary antisera. Finally, the sections were counterstained with Mayer's hematoxylin solution.

6.B.3. Results

The room temperature and humidity of the room where bulls were kept during insulation period and the scrotal temperature and the rectal temperature of the animals are presented in Table 6.B.1. The scrotal temperature was maintained about 2^{o}C below the normal body temperature, which indicate that the induced thermal stress to the testis was mild.

The weekly changes of LH, testosterone and estradiol concentration from first castration until 3 weeks after second castration, and daily changes during the insulation period in both bulls are shown in Fig. 6.B.1. LH concentration did not show a considerable changing pattern in both bulls. With testosterone concentration, Bull A did not show changes after first castration for the next 2 weeks, whereas Bull B showed the reduced concentration following the first castration. Both bulls showed a little rise in testosterone concentration in the next day after scrotal insulation, and the more considerable was the decreasing pattern of testosterone concentration in both bulls as the insulation time was advancing. However, the decreasing pattern was variable among the bulls (beginning of insulation vs. end of insulation in Bull A and Bull B was 6.19 vs. 2.38 and 4.11 vs. 2.07 ng/ml, respectively). There was a rapid decrease in the testosterone level after second castration. The changes of estradiol concentration in both bulls are highly related to the changes of testosterone concentration during the insulation period.

P450scc enzyme was expressed only by the Leydig cells of both the control and insulated testis (Fig. 6.B.2). When compared its expression pattern between the control and insulated testis in both bulls, the insulated testis demonstrated a decreased number of P450scc positive cells with reduced intensity. This result clearly indicated that the

mild thermal stress of the bovine testis for 1 week might cause the downregulation of P450scc enzyme, which in turn may cause the altered steroidogenesis by the insulated testis.

6.B.4. Discussion

The present study is the first of its kind that compares one testis before inducing thermal stress with another contralateral testis of the same animal after inducing thermal stress in order to investigate the effect of increased testicular heat. This kind of experimental model might have reduced the chances of individual variation if different animals are used for control. Furthermore, the scrotal insulation was started only after the complete recovery from the first castration, which took 2 weeks for Bull A and 3 weeks for Bull B. However, the castration stress and the inflammation in the scrotum for few days after the first castration should be taken into consideration while interpreting the results of the present experiment. Since the present result of reduced testosterone synthesis by the testis after increased testicular temperature is almost similar with the previous reports in bull (144, 161) and ram (25, 26), the first castration and its stress might have caused a little influence in the testicular steroidogenesis after 2-3 weeks. This kind of experimental model may be useful to reduce the number of animals and the differences caused by the individual animals used for control.

The present study, for the first time, indicated the downregulation of P450scc enzyme even by a mild thermal insult of the bovine testis for 1 week. Furthermore, the bovine testis under thermal stress produced low testosterone that might have relation with the reduced P450scc activity. The present study demonstrated the immunohistochemical localization of P450scc enzyme in the Leydig cells of both

control and insulated testes, which is consistent with the previous findings in the contralateral scrotal and cryptorchid testis in bull (Chapter 5.B) and in the rat (136), Hokkaido sika deer (76) and Shiba goat (181) testis. The activities of several other enzymes such as 17-α hydroxylase, 17β-hydroxysteroid dehydrogenase (20), 3β-hydroxysteroid dehydrogenase (88) and 17,20-lyase (60) involved in the Δ^4 and Δ^5 testosterone biosynthetic pathways are reported to be heat sensitive. Our present finding, for the first time, indicated the down-regulation of P450scc enzyme in the insulated testis, which is similar as our previous observation in the postnatally developing bovine cryptorchid testis after 8 months of age (Chapter 5.B). Taken together, the exposure of the bovine testis, capable for spermatogenesis, to increased heat may cause the downregulation of P450scc enzyme causing decreased testosterone production. However, it cannot be speculated if such downregulation is reversible, and if there is also the alteration in LH receptor activity as reported earlier in rats (85).

Both bulls used in the present study demonstrated almost similar kind of reducing pattern of testosterone concentration during insulation period. However, it was more pronounced in Bull A than in Bull B. Moreover, Bull A almost maintained the similar testosterone concentration after the first castration, which was decreased in Bull B. This might have caused the difference in patterns between two bulls since the testosterone concentration in both bulls at the end of insulation period was almost similar. Moreover, the interval between the first castration and the insulation in Bull A was shorter as compared to Bull B, and its difficult to speculate if the testosterone concentration in Bull A would have dropped, had it been waited for 1 more week, or the first castration and the inflammation of the scrotum has contributed for lower testosterone in Bull B. Although there may be the bull-to-bull variation for the initial

basal testosterone level, the lowered testosterone concentration after scrotal insulation may be a key indicator related to the testosterone producing capacity of the testis under thermal stress, which may be similar between the bulls. Interestingly, the present result indicated almost similar changing pattern of estradiol as that of testosterone during the insulation period. However, its not clear if such kind of mild thermal insult in the bovine testis for 1 week causes any difference in cytochrome P450 aromatase activity, which is responsible for the conversion of testosterone into estrogen. In conclusion, mild thermal stress of the bovine testis for 1 week may cause the downregulation of P450scc enzyme resulting reduced testosterone synthesis. P450scc enzyme activity in the testis may be heat sensitive.

Table 6.B.1. Environmental conditions and scrotal and rectal temperatures of bulls during 1 week of scrotal insulation period (July 21-28, 2004).

Room temperature (°C)		Humidity (%)	Scrotal temperature during insulation (°C)		Rectal temperature (°C)	
Maximum	Minimum		Bull A	Bull B	Bull A	Bull B
27.9 ± 0.8	19.9 ± 1.0	79.6 ± 1.8	36.5 ± 0.2	36.7 ±0.3	38.9 ± 0.2	38.6 ± 0.2

Data are presented as Mean ± SEM.

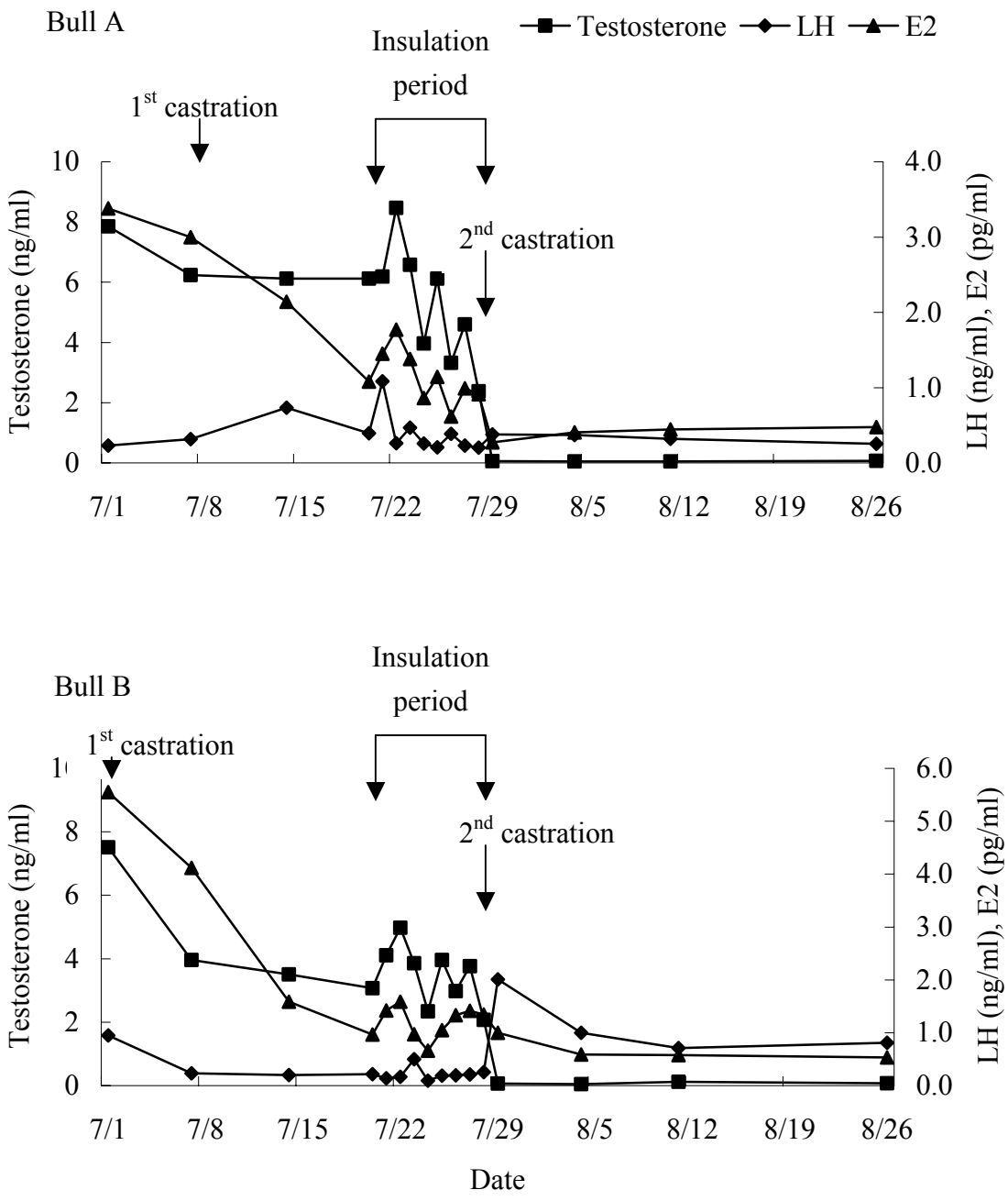

Fig. 6.B.1. Changes in LH, testosterone and estradiol concentrations in Bull A (top) and Bull B (bottom) from before and after unilateral castration, during scrotal insulation for 1 week and after second castration. First castration in Bull A and Bull B was performed on July 1 (7/1) and July 7 respectively. Insulation period was from July 21 to July 28.

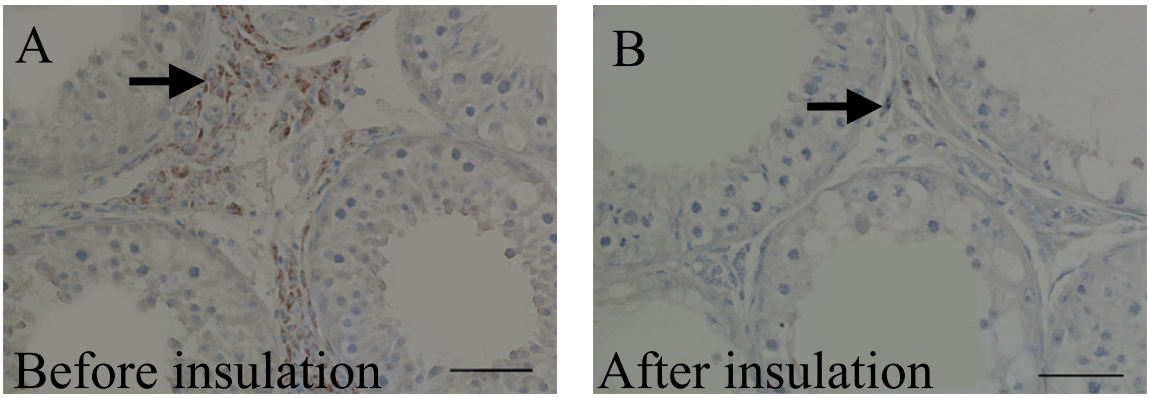

Fig. 6.B.2. Comparison of the expression pattern of P450scc enzyme in the Leydig cells (arrows) of the control testis (A, before insulation) and the insulated testis (B, after insulation) in one representative bull. The number of P450scc positive Leydig cells and the intensity of expression were considerably reduced in the testis after insulation (B). Bars: 100 μm.

Chapter 7

Testicular Pathology and Response of Pituitary-Testicular Axis with GnRH Treatment in Sub-fertile Holstein Bulls

Chapter 7.A

Testicular Histopathology and Alterations in the Immunohistochemical Expression of α-Smooth Muscle Actin (SMA), Vimentin and Cytokeratin

7.A.1. Introduction

The expression patterns of α-smooth muscle actin (SMA) and vimentin in the bovine testis indicate the maturation status of seminiferous tubules (53, 54). Some bulls reared for breeding purpose produce substandard quality of spermatozoa that may cause sub-fertility. Testicular biopsies of men with impaired fertility generally reveal the lesions of mixed atrophy, characterized by the seminiferous tubules having full spermatogenesis up to 'Sertoli cell only' features (22, 162). Although testicular lesions in sub-fertile bulls are not specified so far, some cases of mild to severe spermatogenic lesions in the testis are reported in bulls (69, 119). The expression of cytokeratin in the seminiferous epithelium of the adult human testis with impaired fertility has been regarded as a sign of either maintaining or regaining undifferentiated immature feature (15, 22, 114, 153, 167, 168, 171). Similarly, the Sertoli cells of germ-cell-depleted tubules in the aging human testis express cytokeratin and increased vimentin (52). The present study investigates the pathological lesions of the sub-fertile bull testis, and its relation with the changes in immunohistochemical expression of α-SMA, vimentin and cytokeratin.

7.A.2. Materials and methods

The bulls reared in Genetics Hokkaido (n=4) and National Livestock Breeding Center (NLBC), Nikkappu Station, Hokkaido (n=1) were found having low semen quality after routine evaluation of their semen to measure the semen volume, spermatozoa concentration, motility of spermatozoa after collection and motility of spermatozoa after freezing and thawing. On the basis of low semen quality, as compared to the other bulls, for a prolonged period (at least 6 months), these bulls (n=5) were identified as sub-fertile bulls and decided to remove them from the bull centers. These bulls were used for another experiment to study their response on GnRH treatment (Chapter 7.B), and after several weeks, bilaterally castrated to collect their testes. Testis samples were fixed in Bouin's solution, blocked in paraffin, and sectioned (5 μm) and stained by routine Hematoxylin-eosin (HE) staining method and by specific immunohistochemical staining for α-SMA, vimentin and cytokeratinas described previously (Chapter 2.3-2.5). The primary antibodies used were the mouse monoclonal anti-α-smooth muscle actin (1:5000; Clone No. 1A4, Sigma Chemical Co., St. Louis, USA), polyclonal anti-calf lens vimentin (1:150; MEDAC, Hamburg, Germany) and the rabbit polyclonal anti-keratin (1:100, DAKO, Copenhagen, Denmark). The control sections were treated with normal horse and normal goat serum instead of primary monoclonal and polyclonal antisera, respectively. The sections were counterstained with Mayer's hematoxylin solution.

7.A.3. Results

7.A.3.1. Seminiferous tubule lesions

The sub-fertile bull testes demonstrated different types of spermatogenic lesions

in the adjacent tubules. Some tubules were almost normal, while other tubules had moderate to severe lesions ranging from spermatids to spermatocytes arrests, with some tubules showing 'Sertoli cell only' features (Figs. 7.1A-D). The degree of atrophy in the later type of tubules was considerably high. Based on the similar type of lesions in human infertile or sub-fertile testis termed as mixed atrophy, we believe that this kind of lesions in bovine testis may be a typical testicular mixed atrophy in bovine. Moreover, testes from one bull showed various dysplastic areas characterized by obliteration of tubules and accumulation of spermatozoa (Fig. 7.1C, D). This kind of dysplastic lesions of the testis is not so far reported in bovine.

7.A.3.2. Alterations in the immunohistochemical expressions of α-SMA, vimentin and cytokeratin

Seminiferous peritubular α–SMA expression in the sub-fertile testis demonstrated no considerable change in the Sertoli cell only tubules and tubules with severe spermatogenic arrest (Fig. 7.2.A, right). However, α–SMA expression has been characteristically different in the dysplastic areas (Fig. 7.2.A, left). Sertoli cell vimentin is remarkably increased and altered in its expression pattern in the Sertoli cell only tubules of the mixed atrophied sub-fertile bull testes (Fig. 7.2.B), which is almost similar to a transforming pattern as reported previously (Chapter 3, 5.A). Sertoli cells of the mixed atrophied testes did not express cytokeratin (not shown).

7.A.4. Discussion

Some previous studies have reported the cases of impaired spermatogenesis in bulls (12, 69, 119, 146). These include from Sertoli cell only syndrome to the arrest at

the stage of primary spermatocytes and degenerative spermatocytes. Mixed atrophy in the human testis is described with different types of spermatogenic lesions in the adjacent tubules and termed as mixed atrophy (22, 162). The present histological study of the sub-fertile bull testes clearly demonstrated different types of spermatogenic lesions such as the arrest at the stage of spermatids and spermatocytes and Sertoli cell only syndrome in the adjacent tubules and we propose such lesions as testicular mixed atrophy of the bovine testis. Moreover, the present finding, for the first time, demonstrated a typical case of dysplastic lesions in the mature bovine testis, even though its reason is not clear. The toxicological studies in rats exposed in fetal life to chemical esters of phthalate have shown testicular dysgenesis postnatally (61, 122). These chemicals produce anti-androgenic effect during fetal and early postnatal life but plasma testosterone during maturity remains unaffected (61). The hormonal monitoring of such bulls may also be useful. The cases of bull sub-fertility can be identified easily in the breeding stations by evaluating semen characteristics and studies of such bulls for their testicular pathology and associated endocrine status may have a potential research value.

The present finding revealed no changes in the peritubular α-SMA in the Sertoli cell only tubules of sub-fertile bull testes, as observed previously in the 18-month-old cryptorchid testis with mostly Sertoli cell only tubules (53). The expression pattern of α-SMA in the dysplastic areas observed in this study is almost similar to the expression pattern of α-SMA reported in the dysgenetic areas of the rat testis exposed *in-utero* to the chemical toxicant, dibutyl pthalate (61). This distinct expression pattern of α–SMA can be important to identify the dysplastic lesions in the sub-fertile testis.

The increase in Sertoli cell vimentin immunolabelling has been reported in some pathological (16, 168) and aging human testes (52). Our present finding of increased Sertoli cell vimentin expression in the Sertoli cell only tubules of sub-fertile bull testes is consistent with these findings. Furthermore, this increased vimentin and its expression pattern in the Sertoli cell only tubules are almost similar to a transforming pattern (53, 54), which may indicate the reversion of Sertoli cell maturity in the mixed atrophied bovine testes although its reason is unknown. We suggest this change as a reversion of Sertoli cell maturity, because these bulls had a previous history of having good semen quality and probably had a functioning mature Sertoli cells. In other animal species, cytokeratin is expressed in the adult seminiferous tubules of testis with impaired fertility (15, 22, 114, 153, 167, 168, 171), which is considered as a sign of undifferentiated immature feature of Sertoli cells. However, interestingly, cytokeratin was not expressed in the Sertoli cells only tubules of sub-fertile bull testes. This striking finding may indicate that vimentin expression pattern, not of the cytokeratin, is a reliable indicator of the differentiated Sertoli cells, capable of supporting spermatogenesis in the bovine testis.

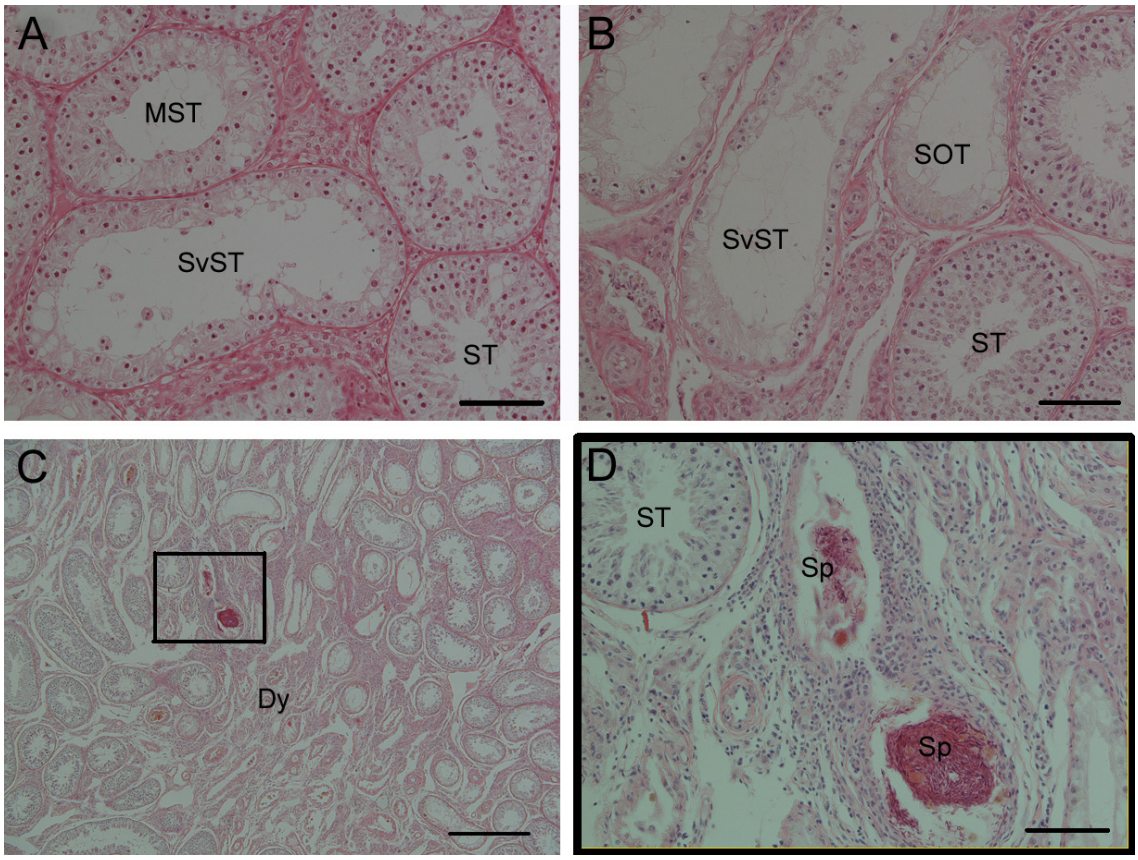

Fig. 7.1. Hematoxylin-eosin stained sections of the sub-fertile bull testes with different types of spermatogenic lesions in the adjacent seminiferous tubules (A, B). Testes from one bull showed various dysplastic areas characterized by obliteration of tubules and accumulation of spermatozoa (C, D). Rectangular area in C is enlarged in D. ST: Normal seminiferous tubule, MST: tubule with mild spermatogenic arrest, SvST: tubule with severe spermatogenic arrest, SOT: Sertoli cell only tubule, Dy: dysplastic area, Sp: spermatozoa accumulation, Bars: A, B, D: 100 µm, C: 500 µm.

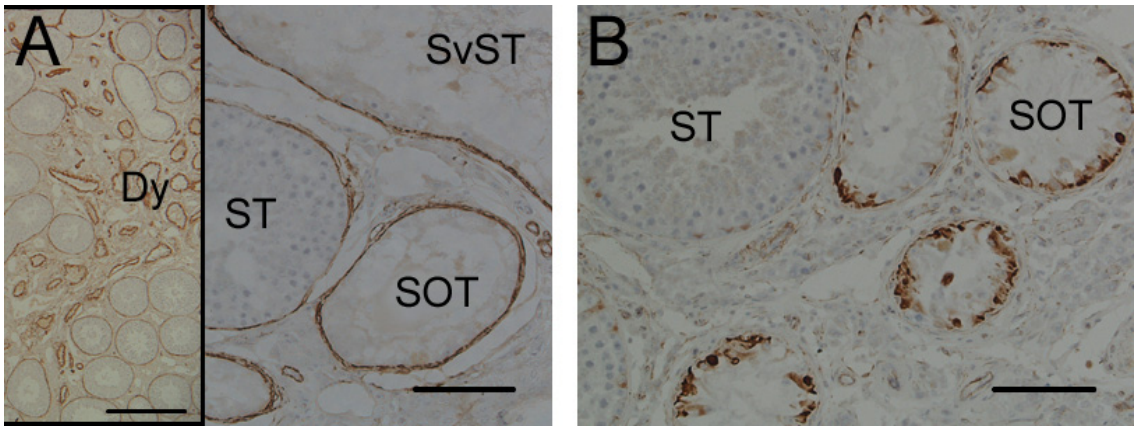

Fig. 7.2. Immunohistochemical expression patterns of α-smooth muscle actin (α-SMA) (A) and vimentin (B) in the sub-fertile bull testes with different types of mixed spermatogenic lesions. Dysplastic areas are characteristically distinguished with the help of α-SMA expression pattern (A, left), but SOT has the similar α-SMA expression as that of the ST. Vimentin expression is remarkably increased in the SOT as compared to STNo ST: normal seminiferous tubule, Dy: dysplastic area, SOT: Sertoli cell only tubule, SvST: tubule with severe spermatogenic arrest. Bars: A (right), B: 100 μm, A (left): 500 μm.

Chapter 7.B

Pituitary and Testicular Response with GnRH treatment

7.B.1. Introduction

Bull fertility is very important because one bull may serve around 20 females under natural service conditions or hundreds of thousands under artificial insemination program. Many bulls produce less or substandard quality of spermatozoa, which makes less number of inseminates, and may also impair fertility post insemination or post service. Different types of spermatogenic arrests leading to impaired fertility and their relationships with gonadotrophic and gonadal steroid hormones are described in men (18, 24, 110), rats (129) and also in bulls (29, 119). Although moderately predictable for the absolute fertility level, semen quality parameters of motility, sperm number and sperm morphology are valuable in identifying bulls of very low fertility (180). The peripheral level of gonadotrophins and testicular steroids may not be related to the testicular pathology in bulls, which points towards other abnormalities, including impaired gonadal responses to those hormones or secretion of intratesticular factors (119). The pituitary and testicular response with exogenous GnRH and its relationship with the semen quality in bulls is not clear. Such response has been reported with no relation on the semen production (2) and libido (35) of bulls. However, testosterone response to GnRH is considered more reliable predictor of bull fertility than other reproductive measurements such as semen quality and SC parameters (143). The present study evaluated the semen quality of bulls for at least 4 months, identified them as bulls with good or poor semen quality and grouped them as control or sub-fertile

bulls, and compared the basal and GnRH stimulated responses in the circulating LH, testosterone and estradiol-17β (E_2) concentrations.

7.B.2. Materials and methods

7.B.2.1. Animals

Actively semen donating bulls reared in Genetics Hokkaido (n=8) and National Livestock Breeding Center (NLBC), Nikkappu station (n=1), Hokkaido, Japan were used in the present study. Both farms are the breeding bull stations where the management practices were almost similar. In both stations, semen was collected once or twice every week, ejaculating 2 to 3 times using artificial vagina, and routinely evaluated for volume, spermatozoa concentration and progressive motilities after collection (MAC) and after freezing into liquid nitrogen and thawing (MAT). Bulls, in Genetics Hokkaido (n=4) and in NLBC (n=1), having less than 60% average MAC or less than 30% average MAT for at least 4 months continuously, were identified as sub-fertile bulls. Out of them, 2 bulls were young (1.5 and 2.5 years old) and other 3 bulls were older (5-7 years). Bulls (n=4) having good semen quality were randomly selected in Genetics Hokkaido, whose ages were 2.5 year (n=1) and 5-7 years (n=3), and used as control bulls. Since the semen quality of young and older bulls differ, the comparison of semen parameters in young (1.5 to 2.5 years) and older (5-7 years) bulls for both groups were carried out separately.

7.B.2.2. Treatment and blood sampling

Bulls in sub-fertile group (n=5) and control group (n=4) were treated intramuscularly once with GnRH (250 μg of Conceral®, Schering-Plough Animal

Health K.K., Tokyo). Blood samples were collected as -1d, -30 min, 0 (time of treatment) followed by every 30 min for 5 h, and 1 d, 3 d and 5 d after treatment. Plasma was separated and harvested immediately at -30^{o}C until assayed to measure LH, testosterone and E_2 concentrations. The first 3 samples before treatment were used to determine the pre-GnRH-treatment or basal concentrations and the 3 samples after 1 d until 5 d of treatment were used to determine the first-five-days response. The hormone concentrations at 0 (time of treatment) was considered as the base line and the percentage increment in every 30 min during 5 h following treatment was determined to investigate the first-five-hours response of GnRH treatment on the plasma concentrations of LH, testosterone and E_2. Since the basal and GnRH stimulated hormone concentrations in young and older bulls were similar, the analysis of hormonal data was carried out only between the sub-fertile and control group, including young and older bulls of the respective group together. LH was measured only for two sub-fertile (S1 and S2) and two control (C1 and C2) bulls, and the bull-wise data are presented for the first-five-hours response. However, testosterone and E_2 were measured for all bulls and group-wise data are presented for the first-five-hours responses.

7.B.2.3. Hormone assay

The plasma LH concentrations were measured by a double-antibody radio-immunoassay (RIA) and testosterone and E_2 were measured by double antibody enzyme-immunoassay (EIA) as described in Chapter 2.6. The intra-assay and inter-assay coefficients of variation (CVs) for LH RIA, testosterone EIA and E_2 EIA were 11% and 8%, 4.7% and 8.7%, and 7.1% and 14.9%, respectively.

7.B.2.4. Statistical analysis

JMP 5.0.1 software was used for statistical analysis and the probability values $P<0.05$ were considered as significant. Data of semen parameters and hormone concentrations were pooled and analyzed by ANOVA. Student's t-test was applied to compare the means between and within the groups at young and older ages. The effect of group and time, and group-time interaction on the testosterone and E_2 increment for the first five hour after GnRH treatment was analyzed by the repeated measure ANOVA. However, LH was measured only for 2 animals from each group and repeated measure ANOVA could not be applied for LH.

7.B.3. Results

7.B.3.1. Comparison of semen parameters

The comparison of semen parameters between young and older, sub-fertile and control bulls are presented in Table 7.B.1 and 7.B.2, respectively. Sub-fertile group had lower than 60% and 30% MAC and MAT, respectively. Only the MAC and MAT in young bulls, and all 4 parameters of semen volume, spermatozoa concentration, MAC and MAT in older bulls differed significantly ($P<0.01$) between sub-fertile and control group. Moreover, both groups showed increase ($P<0.01$) in semen volume during older age. Semen concentration decreased ($P<0.05$), while MAC and MAT increased ($P<0.01$) in sub-fertile older bulls as compared to sub-fertile young bulls. The young control bull had a higher ($P<0.01$) MAT as compared to older control group.

7.B.3.2. Comparison of average pre-GnRH-treatment (basal) and first five days concentrations of LH, testosterone and E_2

The comparison of average pre-GnRH-treatment (basal) and average of first five days concentrations of LH, testosterone and E_2 production is shown in Fig. 7.B.1A and B, respectively. The basal concentrations of these hormones were not significantly different between the groups. Interestingly, the average of first five days concentrations of testosterone and E_2 were significantly ($P<0.01$) lower in the sub-fertile group as compared to the control group.

7.B.3.3. Comparison of the first-five-hour response of GnRH treatment on the plasma concentrations of LH, testosterone and E_2

The bull-wise percentage increase in LH is shown in Fig. 7.B.2. The LH increase in one sub-fertile bull (S2) was interestingly very high and in another bull (S1) was even lower than in the control bulls (C1 and C2). However, the time of peak LH after GnRH treatment was almost similar (between 2-3 hours) in all bulls. The group-wise percentage increase in testosterone and E_2 after GnRH treatment is demonstrated in Fig. 7.B.3. The repeated measure ANOVA showed the significant group effect ($P<0.05$) on testosterone increment and time effect ($P<0.01$) on testosterone and E_2 increment for the first five hour. There was no time-group interaction for both hormone increments. The peak increase of testosterone concentration in both groups was observed at 3 h after the treatment. Interestingly, the testosterone increment in sub-fertile bulls was observed late and was lower in concentrations as compared to the control group, with a maximum difference ($P<0.01$) between 1-3 h and a moderate difference ($P<0.05$) at 0.5 h, 3.5 h, 4 h and 4.5 h of treatment. However, the animal-to-animal variation was higher in sub-

fertile group. The peak increase of E_2 concentration in sub-fertile and control group was observed after 4 and 5 h, respectively, without any difference between the groups.

7.B.4. Discussion

The plasma concentrations of LH and testosterone after GnRH treatment increased rapidly in both the sub-fertile and control groups, as previously reported in bulls (66, 142) and in buffalo bulls (1). However, the difference in LH response between the sub-fertile bulls was very high and its reason was not clear. Moreover, the present results clearly demonstrated that the first-five-hour testosterone increment in sub-fertile group started late and its increment was lower as compared to the control group. Similarly, the first-five-days concentration of testosterone was also lower in the sub-fertile group. Furthermore, the significant group effect observed between the sub-fertile and control group on the first-five-hour testosterone response indicate that the plasma testosterone concentration after GnRH treatment may be related to the semen quality parameters in bulls. This result also supports the previous report that the treatment with GnRH and assessment of plasma testosterone concentrations has some correlation with the semen production in the mature bull (66), and it could be a useful test in the young bull selection for their reproductive potential (142). The basis of grouping bulls as sub-fertile and control in this experiment was based on the sperm motility. A positive correlation was reported in Ayrshire bulls between GnRH-induced testosterone concentration and fertility of bulls with inferior spermatozoa motility (11). Only the microscopic evaluation of sperm motility as a measure of semen quality evaluation may not allow a satisfactory prediction of fertility (43). Therefore, testicular response to produce testosterone may be used as an alternative tool. However, due to

high animal-to-animal variation and less number of animals used in our study, further study in large population may be required to establish a strong relationship.

The present finding of the peak plasma testosterone concentration 3 h after GnRH treatment is consistent with the previous reports in bulls (Post et al., 1987) and in buffalo bulls (Abdel-Malak et al., 1992). This is probably the first report that has also evaluated the E_2 response in bulls after GnRH treatment. The peak E_2 increment after GnRH treatment was observed in 4 h in sub-fertile group, while it was increasing until 5 h in control group. The time interval between testosterone peak and E_2 peak may suggest the time required for the aromatization of testosterone into E_2. Interestingly, a significant difference in the E_2 concentration between the sub-fertile and control group was observed for the first five days after GnRH treatment. It suggests that the concentration of E_2 may be related to the concentration of testosterone in the mature bulls.

The testicular pathology of these bulls demonstrated mixed atrophic lesions and several seminiferous tubules were Sertoli cell only tubules, without germ cells, and Sertoli cells showed immature undifferentiated pattern of vimentin expression (Chapter 7.A). It can be speculated that the weak responses by such pathological testis on testosterone synthesis after GnRH treatment might be due to altered LH receptors or their activities that may alter testicular steroidogenesis. Further study may be required in this aspect.

In conclusion, peak testosterone increment in bulls can be assessed around 3 h after GnRH treatment. Sub-fertile bulls may have a slow and weak response on testosterone production after GnRH treatment, which may also indicate their semen quality status.

Table 7.B.1. Comparison of semen parameters between sub-fertile and normal (control) bulls between 18-30 months of age.

Semen parameters *	Sub-fertile bulls (n=2)	Control bulls (n=1)
Semen volume (ml)	7.3 ± 0.5	6.2 ± 0.4
Spermatozoa concentration (x10^6/ml)	614.3 ± 31.3	793.5 ± 62.3
Progressive motility after collection (+++%)	44.5 ± 2.4 [a]	72.8 ± 1.2 [b]
Progressive motility after freezing and thawing (+++%)	9.4 ± 1.3 [a]	49.0 ± 1.8 [b]

*: Semen parameters evaluated for 20.5 and 10 collection days in sub-fertile and control bulls, respectively. Data are expressed as Mean ± SEM. Different superscripts between the groups denote significant differences (P<0.01).

Table 7.B.2. Comparison of semen parameters between sub-fertile and normal (control) bulls between 5-7 years.

Semen parameters *	Sub-fertile bulls (n=3)	Control bulls (n=3)
Semen volume (ml)	11.8 ± 0.3 [a]	16.2 ± 0.4 [b]
Spermatozoa concentration (x10^6/ml)	486.4 ± 26.7 [a]	947.2 ± 46.4 [b]
Progressive motility after collection (+++%)	57.6 ± 0.6 [a]	70.3 ± 0.6 [b]
Progressive motility after freezing and thawing (+++%)	26.9 ± 1.2 [a]	37.0 ± 0.8 [b]

*: Semen parameters evaluated for 27±4.9 and 30.0±8.3 collection days in sub-fertile and control bulls, respectively. Data are expressed as Mean ± SEM. Different superscripts between the groups denote significant differences (P<0.01).

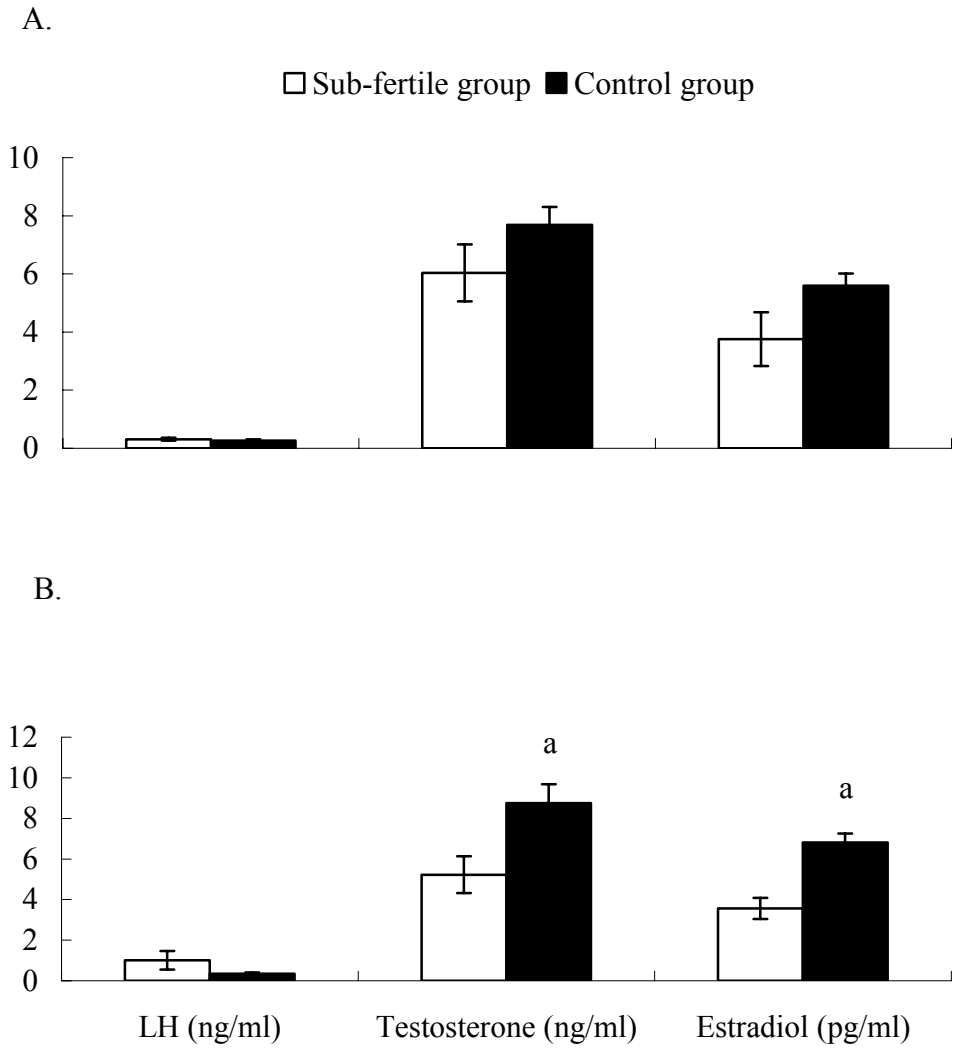

Fig. 7.B.1. Comparison of average pre-GnRH-treatment (basal) (A) and average of first five days (B) concentrations of LH, testosterone and estradiol (E_2) in sub-fertile and control groups. Pre-GnRH-treatment concentrations are the mean concentrations of –1 d, -30 min and 0 min (treatment time) and first five days concentrations are the mean concentrations of 1 d, 3 d and 5 d after treatment. Significant differences between the groups are denoted as a: $P<0.01$.

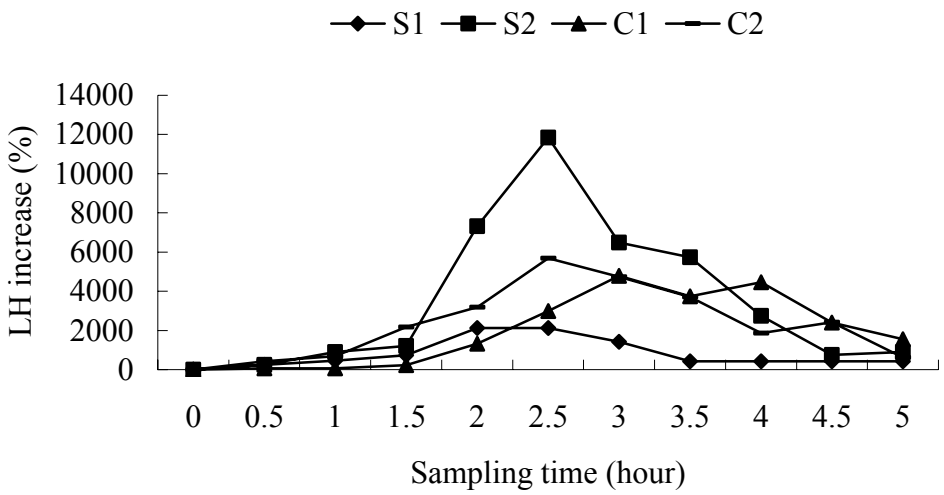

Fig. 7.B.2. First-five-hour response of GnRH treatment on plasma LH concentration in sub-fertile (S1 and S2) and control (C1 and C2) bulls. Sampling schedule: 0 h (treatment time) followed by every 30 min for 5 h.

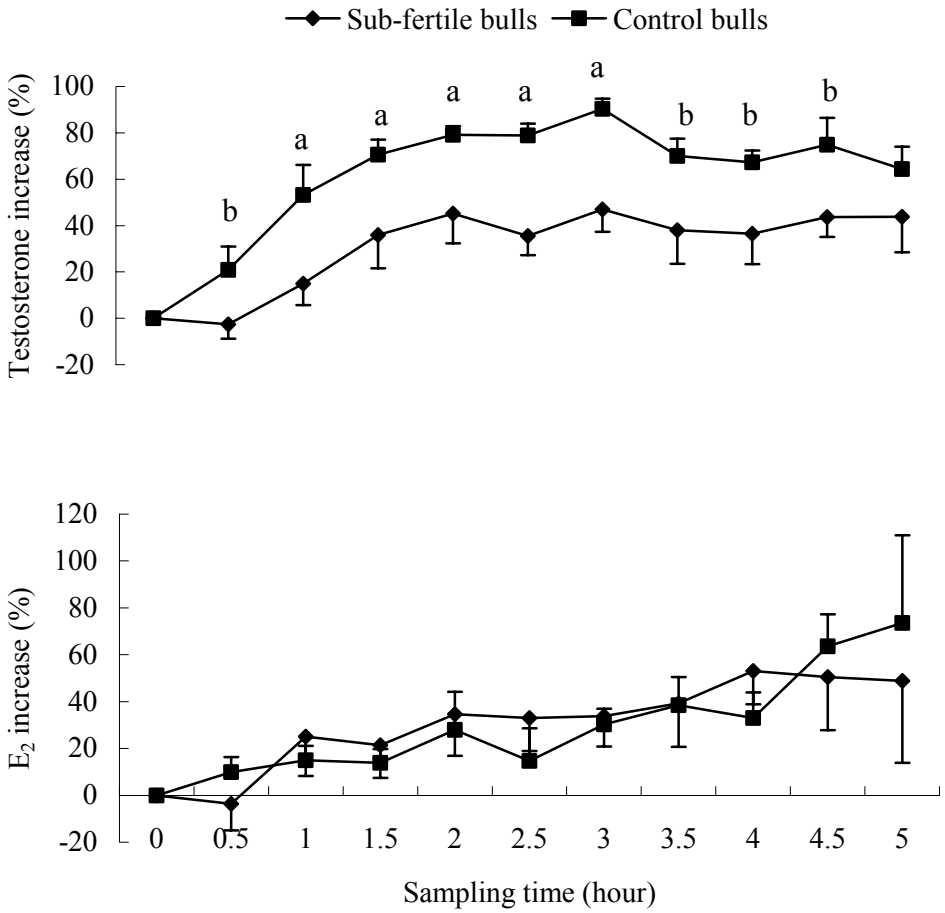

Fig. 7.B.3. First-five-hour response of GnRH treatment on plasma testosterone and estradiol (E_2) concentrations in sub-fertile and control groups. Sampling schedule: 0 h (treatment time) followed by every 30 min for 5 h. Significant differences between the groups are denoted as a: $P<0.01$ and b: $P<0.05$.

Chapter 8

Summary and Conclusion

The present study on the reproductive functions in bulls focused on the postnatal developmental regulation of seminiferous tubules and Sertoli cells, functional regulation of spermatogenesis in pubertal and postpubertal bulls, developmental alterations due to increased heat and pathological lesions in the testis, altered steroidogenesis in the testis caused by increased heat, and the pituitary-testicular response in sub-fertile bulls after GnRH treatment.

1) The rapid increase in the seminiferous tubule diameter and the transformation and maturation of Sertoli cells are the major events in the postnatally developing testis. Failure of these events may lead to the failure of spermatogenesis. However, the specific maturation markers of seminiferous tubules and Sertoli cells are not understood in the postnatally developing bovine testis. The present study demonstrated that the seminiferous peritubular specific α-SMA, a cytoskeletal protein, appears around 4 months of age that coincides with the initiation of postnatal division of spermatogonia in the bovine testis. Moreover, the Sertoli cell maturation before than puberty is a crucial process, during which the cells undergo transformation and basal repositioning of the nuclei. During this process, beside than the hormonal support, a special structural support to Sertoli cells may be required, and the role of Sertoli cell specific cytoskeletal proteins such as cytokeratin and vimentin could be important. Cytokeratin is considered as a marker specific to Sertoli cell maturation in many animal

species. The present study demonstrated that cytokeratin is expressed only in the Sertoli cells of early fetal testis in bovine. On the other hand, the three distinct expression patterns termed as the immature, transforming and mature patterns of Sertoli cell vimentin were observed during postnatal development of the bovine testis. It was, therefore, suggested that these specific expression patterns of Sertoli cell vimentin might be useful to understand the maturation status of Sertoli cells postnatally. Moreover, the basal to infranuclear connection of vimentin during Sertoli cell transformation and maturation from around 5 months to 8 months of age, observed in this study, may act as a key structural support to stabilize the Sertoli cell nucleus. The present findings suggested that α-SMA and vimentin could be used as specific maturation markers of the postnatally developing seminiferous tubules and Sertoli cells, respectively, and vimentin may help to stabilize the Sertoli cell nuclei during maturational transformation in the bovine testis.

2) One of the important aspects of the male animal reproduction is the hormonal regulation of spermatogenesis. A lot has been studied in this aspect in the prepubertal bulls. However, the hormonal regulation of spermatogenesis, particularly in postnatal bulls, requires further study. Obtaining good quality spermatozoa postpuberty have tremendous merits in breeding bulls. During microscopic evaluation of the semen, motility has been considered as one of the important determinant of the spermatozoa quality. However, the regulating factors of the spermatozoa motility are not fully understood. Recent studies in other animal species have shown good

evidence about the role of estrogens on spermatozoa motility. Interestingly, the date presented in the present study revealed a significant positive correlation of spermatozoa motility with circulating estradiol-17β (E_2) concentration in postpubertal bulls. The present data, therefore, suggested that E_2 might regulate the spermatozoa motility, *in vivo*, in postpubertal bulls.

3) The expression patterns of α-SMA, vimentin and cytokeratin, as the maturation markers of the bovine seminiferous tubules, were applied in the postnatally developing cryptorchid testis and in the mature testis with mixed atrophic lesions. Histologically, cryptorchid testis revealed immature Sertoli cells, and mixed atrophied testis revealed several kinds of seminiferous tubule lesions ranging from mild spermatogenic arrest to the Sertoli cells only tubules. In both cases, vimentin expression demonstrated a pattern similar to transforming pattern. However, cytokeratin was not expressed in both conditions. This was the first observation in the bovine testis that explained immaturity of Sertoli cells under increased heat caused by cryptorchidism during postnatal development might have been related to the failure of Sertoli cell vimentin to attain a mature pattern, and the mixed atrophied seminiferous tubules also have undifferentiated Sertoli cells. Such immature and undifferentiated Sertoli cells cannot support spermatogenesis, and may cause the failure of spermatogenesis.

4) Germ cells are highly sensitive to increased testicular temperature. Studies have been conducted in the past about the effects of increased testicular temperature on the steroidogenic functions of the testis. The present study in bulls, using cryptorchid testis and scrotal insulation models to study the

effects of heat on the testis, clearly demonstrated that increased testicular temperature decreases the production of testosterone, which was also consistent with the previous reports in other species. The present study further demonstrated that the increased heat in the testis might cause the downregulation of cytochrome P450 side chain cleavage (P450scc), the steroidogenic enzyme that converts cholesterol into pregnenolone in the testosterone synthesis pathway, in the testis. It was, therefore, suggested that the cause of the reduced testosterone synthesis by the testis after increased heat might be due to downregulation of this enzyme. Moreover, the increasing aromatase, the enzyme that converts testosterone to estrogens, in the Sertoli cells with increased age was also observed in the cryptorchid testis. These findings may indicate the functional alterations of the bovine testis, if exposed to increased heat.

5) In another study, mature bulls were grouped into sub-fertile (bulls with poor semen quality) and control (bulls with good semen quality) groups, on the basis of the percentage of progressively motile spermatozoa. These bulls were treated once with GnRH and analyzed for their pituitary testicular responses to produce LH and testicular steroids such as the testosterone and E_2. Previous studies had attempted this kind of response in young bulls and indicated its usefulness on early bull selection. The present study design may be the first of its kind in the mature bulls to compare the responses in poor semen producing and good semen producing bulls. Although, the results indicated high bull-to-bull variation, a significant group effect on testosterone production during the first five hour of GnRH treatment was

observed, in which sub-fertile group had a slow and lower increment of testosterone. Moreover, the average of first five days concentrations of testosterone and E_2 were significantly lower in the sub-fertile group as compared to the control group. The testis of such infertile bulls had mixed atrophied lesions. This finding may help to understand the relationship between the testicular mixed atrophy and compromised response by such testis to the GnRH treatment. It can be suggested that sub-fertile bulls might have a slow and weak response on testosterone production after GnRH treatment, which may also indicate their semen quality status.

The present study only investigated the qualitative differences of the cytoskeletal proteins and steroidogenic enzymes during postnatal development, and under increased heat in the testis. Therefore, it was not possible to specify whether the changing pattern of vimentin expression and its alteration, and the downregulation of P450scc enzyme in the testis under increased heat also have the quantitative difference. Further studies, may be the quantitative analysis using molecular techniques, are required to quantify these differences. Furthermore, the number of animals used in some experiments of the present study was not sufficient for further statistical analysis. However, it is expected that the results of the present study will help to understand more about the normal and altered reproductive functions in bulls, and will serve in the future endeavor of reproductive research of this very important food animal.

雄牛の繁殖機能に関する研究

デブコタ ブミナンド

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雄動物は産子の遺伝的能力に関して雌と同等に寄与しているので，家畜の遺伝的改良は優秀な雄に由来する凍結精液と人工授精の普及および胚移植を介して進んでいる。従って，① 出生後の精細管やセルトリ細胞の構造の調節機構，② 性成熟前後の精子形成の調節機構，③ 精巣の発達と機能に及ぼす暑熱ストレスの影響，および ④ 精子活力の低下の要因解明は優秀な種雄牛の選抜および繁殖にとって究明すべき重要な課題である。

そこで，本研究では ① 種雄牛における出生後の精細管やセルトリ細胞の構造の調節機構，② 性成熟後の精子形成に及ぼす調節機能，③ 精巣内温度上昇と病理学的障害による精巣発育異常，④ 温度上昇下の精巣におけるステロイドホルモン合成異常，および ⑤ 精液性状の不良な種雄牛における GnRH 刺激後の下垂体–精巣の反応性に焦点をおいて研究した。

【１】精巣の出生後の発達過程において，精細管直径の増大およびセルトリ細胞の形態的変化と成熟は主要な事象である。しかし，出生後の精巣内における精細管およびセルトリ細胞の成熟の特異的な指標はまだよく分かっていない。研究の結果，生後 4 か月頃から曲精細管周囲における細胞骨格蛋白の一種である α–SMA の特異的な発現が精祖細胞の出生後の分化開始時期と一致している

ことを認めた。さらに，セルトリ細胞の成熟過程では形態的変化と核基底部への再配置が認められた。この過程においてセルトリ細胞特異的細胞骨格蛋白の一種である cytokeratin と vimentin が重要であることを認めた。今回の研究で cytokeratin は胎子期の早期のセルトリ細胞でのみ発現し，出生後の精巣では発現していなかった。これに対して出生後の vimentin 発現パターンには，成熟過程に特異的な様相を示していて，この特異的発現パターンはセルトリ細胞の成熟性を知る上で有用と思われた。さらにセルトリ細胞の核の安定性にとって，核が形態的変化の過程で基底部へ移動することが重要であることを認めた。これらの結果から，牛精巣の出生後の発達過程において，α-SMA と vimentin は精細管とセルトリ細胞の特異的な成熟の指標として有用と思われた。

【２】雄動物の生殖にとって重要な要因は内分泌的調節を含む精子形成の正常な調節機構である。しかし，性成熟前後の雄牛の精子形成に及ぼす内分泌的調節機構に関してはよく分かっていない。種雄牛の性成熟直後に精子活力の良否を判定することは，その選抜時期を弱齢時に実施する上で望ましい。現在精子活力を顕微鏡下で評価する方法がその良否の最適な指標となっている。しかし，精子活力を調節している要因については雄牛ではよく分かっていない。近年，牛以外の動物では精子活力にはエストラジオール 17β（E_2）が関与していることが示されている。今回の研究で，性成熟後の種雄牛において精子活力と E_2 濃度との間に相関が見い出されたことは非常に興味深いものであった。

【３】潜在精巣と繁殖能の低い種雄牛の精巣において，異常なα-SMA，vimentin および cytokeratin の発現パターンが精細管およびセルトリ細胞の成熟性とどのように関係するか知る目的で調べた。組織学的には潜在精巣では精細

管およびセルトリ細胞の発育は未熟であり，繁殖能の低い種雄牛では様々な精子形成障害像，なかにはセルトリ細胞のみからなる精細管が認められた。潜在精巣の精細管ではα-SMA は 8 か月齢までその発現が認められなかった。さらに両例において vimentin 発現パターンは，未分化な形質転換パターンと類似するものであった。cytokeratin は両例でその発現を認めなかった。これらの結果，精巣の出生後の発達過程において温度上昇下にあるセルトリ細胞の未熟性はセルトリ細胞の vimentin が発現できないことと関係があること，繁殖能の低い種雄牛の精細管ではセルトリ細胞が未分化状態に止まっていることを示すことを始めて明らかにした。

【４】精巣内の温度の上昇は精細胞にとって致死的である。これまでに精巣のステロイドホルモン合成に及ぼす温度上昇の影響について調べられている。今回の研究では自然発生例である潜在精巣と実験的に作出した「保温」精巣において精巣内温度上昇はテスチステロンの産生を減少させることを認めた。さらに精巣内温度上昇はテストステロン合成経路中のコレステロールからプレグネノロンへの変換酵素である P450scc のダウンレギュレーションを引き起こす可能性があることを認めた。これらの結果を踏まえて，精巣内温度上昇によるテストステロン合成阻害は P450scc 酵素のダウンレギュレーションによるものと推察した。また，潜在精巣におけるテストステロンからエストロジェンへの変換酵素である P450 芳香化酵素活性の上昇は潜在精巣における機能的異常の指標となることを認めた。

【５】精液性状が不良な雄牛における GnRH 刺激後の下垂体−精巣の反応性を調べるために，性成熟種雄牛を精子活力が 60%以下の低繁殖群と 60%以上の

正常群に区分した。これらの牛に GnRH を投与して GnRH 刺激後の血中 LH, テストステロンおよび E_2 濃度を測定した。GnRH 刺激後の反応性には個体ごとに差があったが，低繁殖群ではテストステロンの上昇の程度は緩慢で低かった。また，組織学的にはこれらの牛の精巣は様々な精子形成障害を示していた。これらの成果は精巣の障害と GnRH 刺激の反応性との相関を知る上で有用と思われた。

以上のように，今回の研究の結果は種雄牛における正常および異常な繁殖能を解明する上で有用なものであるとともに，将来の家畜の生殖研究の推進に資するものと思われた。

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