Original Article

Study of testicular immunolocalization of inhibin subunits and epididymal histological structures among different antler status in rusa deer (*Rusa timorensis*)

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Abstract

The number of rusa deer (*Rusa timorensis*) raising farms has highly increased in Thailand. Basic data on the reproduction of rusa deer are essential for improvement in deer farming reproduction and the assisted reproductive technologies (ARTs) of endangered deer species. This study aimed to determine the relation of antler changes to testicular and epididymis histological structure and cellular source of testicular inhibin subunits in rusa deer. Eighteen rusa deer testes and epididymis were collected from a slaughterhouse located in Kamphaeng Saen, Nakhon Pathom, Thailand. The male rusa deer used in this study were separated into three groups depending on antler stages (velvet, intermediate and hard antlers). Histological observations of the testes and epididymis were performed. The testes sections of three different antler stages were immunostained by polyclonal antisera raised against inhibin α, β_A and β_B subunits. Means (±SEM) of testicular weight and seminiferous tubule diameter in the hard antler group (76.25±9.80 g and 256.10±7.28 µm) were significantly higher than those of the velvet antler group (40.86±7.41 g and 197.25±7.23 µm) ($p<0.05$). The epididymis of the intermediate and hard antler groups was filled widely with numerous spermatozoa, which, in contrast, were sporadically observed in the velvet antler group. In addition, the inhibin α and inhibin β_A subunits were expressed in both Sertoli cells and Leydig cells while the inhibin β_B subunit was only expressed in Leydig cells. This study suggests that the rusa deer testes secrete inhibin in all antler stages. The present study also clearly demonstrates that rusa deer antler changes are related to testicular weight, seminiferous tubule diameter and epididymal spermatozoa, indicating that antler change is a useful indicator for testicular function in rusa deer.

Keywords: epididymis, immunohistochemistry, Rusa timorensis, testis

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Introduction

In Thailand, there are six species of the family Cervidae, which are categorized as the preserved or protected animals according to the Wild Animal Preservation and Protection Act, B.E. 2535 (1992) of Thailand. Rusa deer (*Rusa timorensis)* has been introduced to many countries in the Indo-Pacific region, including Thailand, for farming (Vos, 1982; Huthawattana, 2008). Thus, understanding the reproduction of rusa deer will economically benefit deer farming operations in Thailand. Moreover, study of the non-endemic Cervidae could lead to profound knowledge about the endangered species. Rusa deer is a seasonal breeder, thereby seasonal effects influence its reproduction (Gizejewski, 2004). The battle of male deer is often seen during the breeding season by fully hard antlers, to attract others and assert dominance (Lincoln, 1973). The annual antler cycle belongs to the secondary sex characteristic, which is controlled by seasonality and circulating testicular hormones (Bubenik, 1983; Bubenik, 1990; Bubenik, 2006; Goss, 1983; Fennessy and Suttie, 1985; Rolf and Fischer, 1990; Sempere, 1990). In our previous study, it was demonstrated that the antler growth in male rusa deer was related to the physiological role of testosterone (Phraluk et al., 2015). Gonadotropin releasing hormone (GnRH) vaccination in male rusa deer resulted in increase in GnRH antibody titer, which negatively correlated with circulating testosterone levels and testes size. It was clearly observed that the rusa deer treated with GnRH vaccine maintained velvet antlers and low fresh semen quality with azoospermia pattern. However, a histological study of rusa deer testes at different antler stages has not yet been reported.

Inhibin is synthesized by the members of transforming growth factor β (TGF-β) and is first recognized as a marker of testicular function (De Jong, 1988; Monfort et al., 1993[a]; Monfort et al., 1993[b]; Medan et al., 2006; Medan et al., 2007). Inhibin is a heterodimeric protein consisting of an α subunit linked by disulfide bridge to one of the two β subunits, β_A or β_B, to form inhibin A or inhibin B. It is generally accepted that in male, the inhibin subunits are supposed to have paracrine and autocrine roles in the regulation of testicular steroidogenesis and spermatogenesis in addition to suppression of follicular-stimulating hormone (FSH) secretion in mammals (Vale et al., 1986; Vale et al., 1988; Burger and Igarashi, 1988, Robert et al., 1989; Shaha et al., 1989; Sanford et al., 2002). Three inhibin subunits are secreted by Sertoli cells and Leydig cells located in the testes. The expression and secretion of inhibin subunits vary along with the stage of the seminiferous cycle (Bhasin et al., 1989) and seasonal changes (Matsubayashi et al., 1991; Nagata et al., 1998; Kawazu et el., 2003; Medan et al., 2006; Weng et al., 2006[a]; Weng et al., 2006[b]; Sheng et al., 2008; Dhakal et al., 2011). The testicular morphology, histology and immunolocalization of inhibin subunits have not yet been defined in rusa deer.

In the present study, the functional relationship between histological structure of testes and epididymis, and localization of testicular inhibin subunits was investigated among the different antler stages in rusa deer. Results could serve as a model to examine spermatogenesis of seasonal breeder, especially in deer species of tropical climate zone.

Materials and Methods

Animals and sample collection: Eighteen testes and epididymis of rusa deer were obtained at a slaughterhouse located in Kamphaeng Saen, Nakorn Pathom province, Thailand. All adult males were separated into three groups depending on the antlers; velvet antlers (A), intermediate antlers (B) and hard antlers (C) (Fig. 1). The classification of antler type was based on Ungerfeld et al. (2008); velvet antlers are defined as growing antlers which are covered with a specialized type of hairy skin, intermediate antlers are antlers which lose their hairy skin and hard antlers are antlers which are fully calcified. Testicular weight was measured using scales. Testes and epididymis were immediately fixed in neutral buffered formalin.



Figure 1 Different antlers of male rusa deer in velvet (A), intermediate (B) and hard (C) stages

Histology: The testicular samples were dehydrated in an automatic tissue processor (Shandon™ Citadel 1000; Thermo Fisher Scientific Inc., DE, USA) and embedded in paraffin block (Shandon™ Histocenter2; Thermo Fisher Scientific Inc., DE, USA). Serial sections cut at 4-6 μm by microtome (Shandon™ AS 325; Thermo Fisher Scientific Inc., DE, USA) were mounted on slides. The sections were deparaffinized by xylene and ethanol. Some sections were stained with hematoxylin-eosin (H&E) for observations of general histology. The seminiferous tubules and epididymis of eighteen rusa deer (36 tissue slides) were histologically

evaluated using an Olympus photomicroscope with a 40× objective lens. A movable cursor dot (approximately 0.1 µm in diameter) was used for measuring the diameter of seminiferous tubules. Jandel Scientific Sigma Scan® Image Analysis software (Jandel Scientific Corporation, Montgomeryville, PA) was used for processing measurements.

Immunohistochemistry: After being deparaffinized with xylene, the tissue sections were incubated to quench nonspecific staining in 3% H_2O_2. The sections were subjected to antigen retrieval by autoclaving and then incubated with primary antibody (1:1000 or 1:3000) raised against porcine inhibin α chain (1-30)-NH_2 conjugated to rabbit serum albumin, rabbit porcine inhibin/activin β_A (81-113)-NH_2 (#305-24D), and cyclic acetyl human inhibin/activin β_B (81-113)-NH_2 (#305-25D). The [Tyr30]-porcine inhibin α chain (1-30)-NH_2 conjugated to rabbit serum albumin ([Tyr30]-porcine inhibin α chain (1-30)-NH_2 was kindly provided by Dr. N. Ling, Neuroendocrine Inc, San Diego, CA, USA. Antisera against cyclic inhibin/activin β_A (81-113)-NH_2 (#305-24D), and cyclic inhibin/activin β_B (81-113)-NH_2 (#305-25D) were kindly provided by Dr. W. Vale, The Salk Institute for Biological Studies, La Jolla, CA, USA. After incubation with specific antibody, the sections were incubated with a secondary antibody, 0.25% (v/v) biotinylated goat anti-rabbit secondary antibody, in PBS for 30 minutes at 37°C, followed by incubation with 2% (v/v) avidin-biotin complex (Vectastain ABC kit; Vector Laboratory, CA, USA). The reaction was visualized with 30 mg 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemica Co, St. Louis, MO, USA) in 150 ml of 0.05 M Tris-buffered saline (pH 7.5) and 30% H_2O_2. Finally, the reacted sections were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The control sections were treated with normal rabbit serum instead of primary antisera.

Statistical analysis: The testicular weight and seminiferous tubule diameter were compared by the two-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. Data were presented as mean ± standard errors (SEM). An analysis of testicular weight and seminiferous tubule diameter was performed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, NC, USA). Values of $p<0.05$ were considered statistically significant.

Results

Testicular weight and seminiferous tubule diameter: The testes were oval shape and surrounded by tunica albuginea. The epididymal structure was coiled tubule. The tubule was divided into 3 regions: caput, corpus and cauda. The size of the testes in the hard antler group was apparently much larger than that of the velvet antler group. The antler changes in carcass and testicular weight in rusa deer are shown in Table 1. There was a significant difference ($p<0.05$) in testicular weight between the hard antler group (testicular weight, 76.25±9.80 g) and the velvet antler group (testicular weight, 40.86±7.41 g), while the carcass weight was not significantly different ($p>0.05$) among the different antler stages. The seminiferous tubule diameter in the velvet antler group (197.25±7.23 µm) was significantly lower than those in the intermediate (236.86±4.10 µm) and hard (256.10±7.28 µm) antler groups.

Table 1 Antler changes in carcass weight (A) testicular weight (B) and seminiferous tubule diameter (C) in rusa deer

Measurement	Velvet antler group (n=7)	Intermediate antler group (n=7)	Hard antler group (n=4)
Carcass weight (kg) [Range]	22.37±1.29[a] [16-27]	21.93±1.25[a] [16-26]	19.85±0.93[a] [17-22]
Testicular weight (g) [Range]	40.86±7.41[a] [23-65]	64±7.41[a,b] [32-91]	76.25±9.80[b] [54-113]
Seminiferous diameter (µm) [Range]	197.25±7.23[a] [118-366]	236.86±4.10[b] [151-382]	256.10±7.28[b] [154-434]

[a, b] Different superscript letters between means within row indicate significant differences ($p<0.05$).

Histology: The H&E stained sections showed many seminiferous tubules including a large amount of germ cells, Sertoli cells, and interstitial cells (Leydig cells) (Fig. 2). In the velvet antler group, a few spermatocyte and spermatid were observed in the seminiferous tubules (Fig. 3). The total spermatogenic cells from spermatogonia to spermatozoa were observed in the hard antler group (Fig. 2).

The epididymal structure was coiled tubule which could be divided into 3 regions: caput, corpus and cauda. The epididymal epithelium type was pseudostratified columnar with cilia. It consisted of several cell types including principal, basal, halo and apical cells (data not shown). The three typical regions of epididymal epithelium in the different antler groups are shown in Figure 3. In the caput region, compared to the other regions, the highest principal cells were located near the basement membrane. The corpus region had more basal cells than the other regions. The principal cells in this region were enclosed. The cauda region presented the lowest columnar epithelium with short cilia. The smallest principal cells were found in this region. In the epididymis, a large amount of epididymal spermatozoa were also found in the hard antler group. In the intermediate and hard antler groups, their epididymis was filled widely with numerous spermatozoa, whereas in the velvet antler group, spermatozoa were sporadically observed.

Immunohistochemistry: In all the testes examined in this study, inhibin α subunit positive-staining cells were observed in the seminiferous tubules and Leydig

cells (Fig. 4). Immunohistochemical localization of inhibin β_A subunits was observed in the same cells as that of the inhibin α subunit, whereas inhibin β_B was observed only in the Leydig cells of all antler stages (Fig. 4). However, the staining of three inhibin subunits tended to increase from the velvet antler group to the hard antler group. Immunoreactivity was not observed for the inhibin subunits in germ cells in all antler stages. No immunostaining was detected in the control sections when normal rabbit serum was stained for the primary antibody.

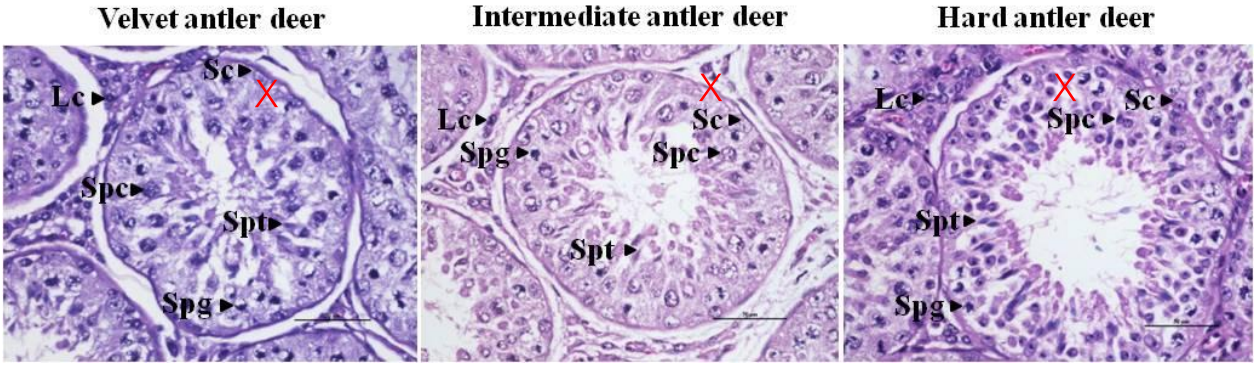


Figure 2 Testicular histology of velvet, intermediate and hard antler rusa deer (SPG, Spermatogonia; SPC, Spermatocyte; SPT, Spermatid; SCs, Sertoli cells; LCs, Leydig cells; 400× magnification, bars indicate 50 µm)

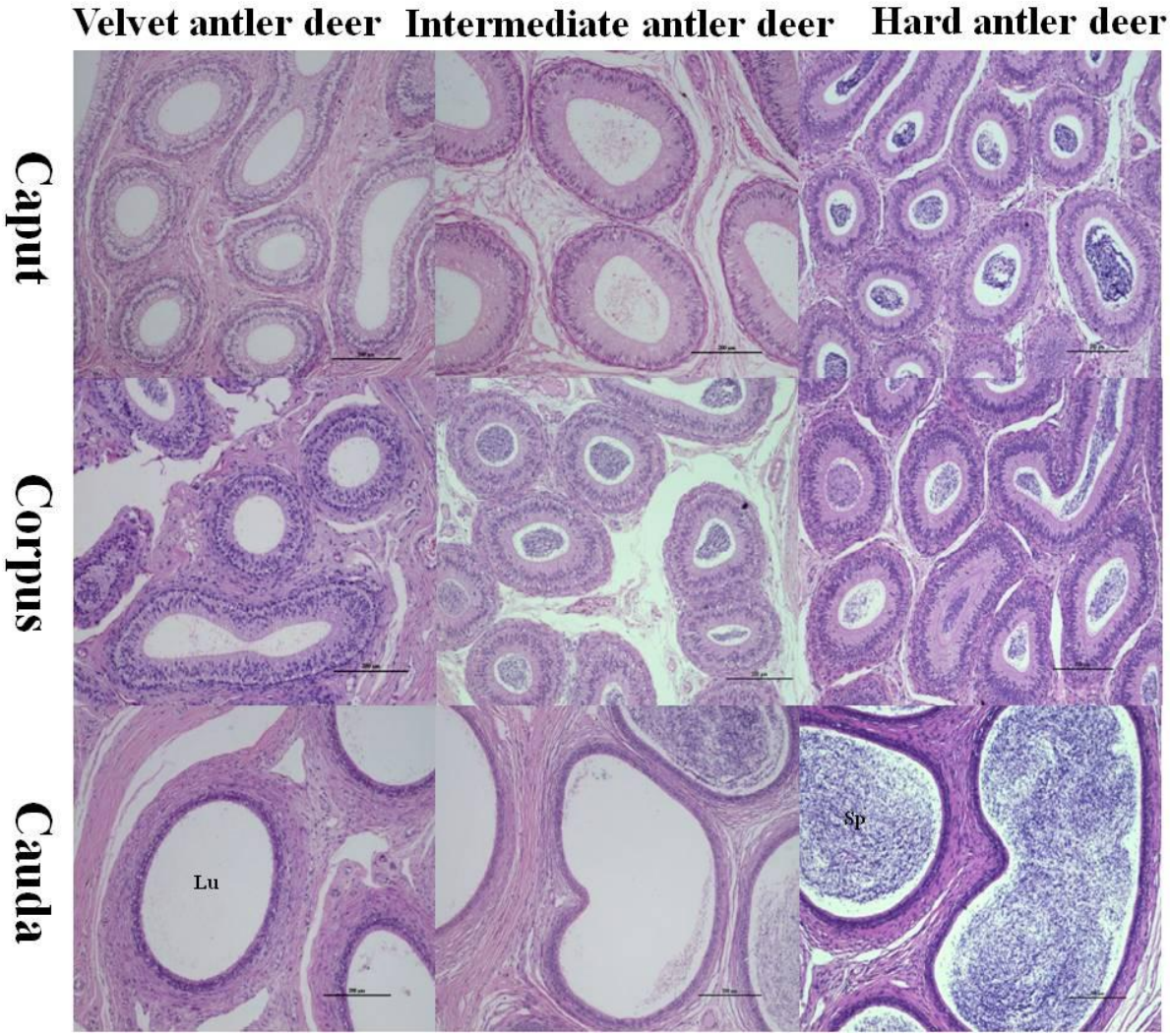


Figure 3 Three regions (caput, corpus and cauda) of epididymis of velvet, intermediate and hard antler rusa deer (LU, Lumen; Sp, Spermatozoa; 100× magnification, bars indicate 200 µm)

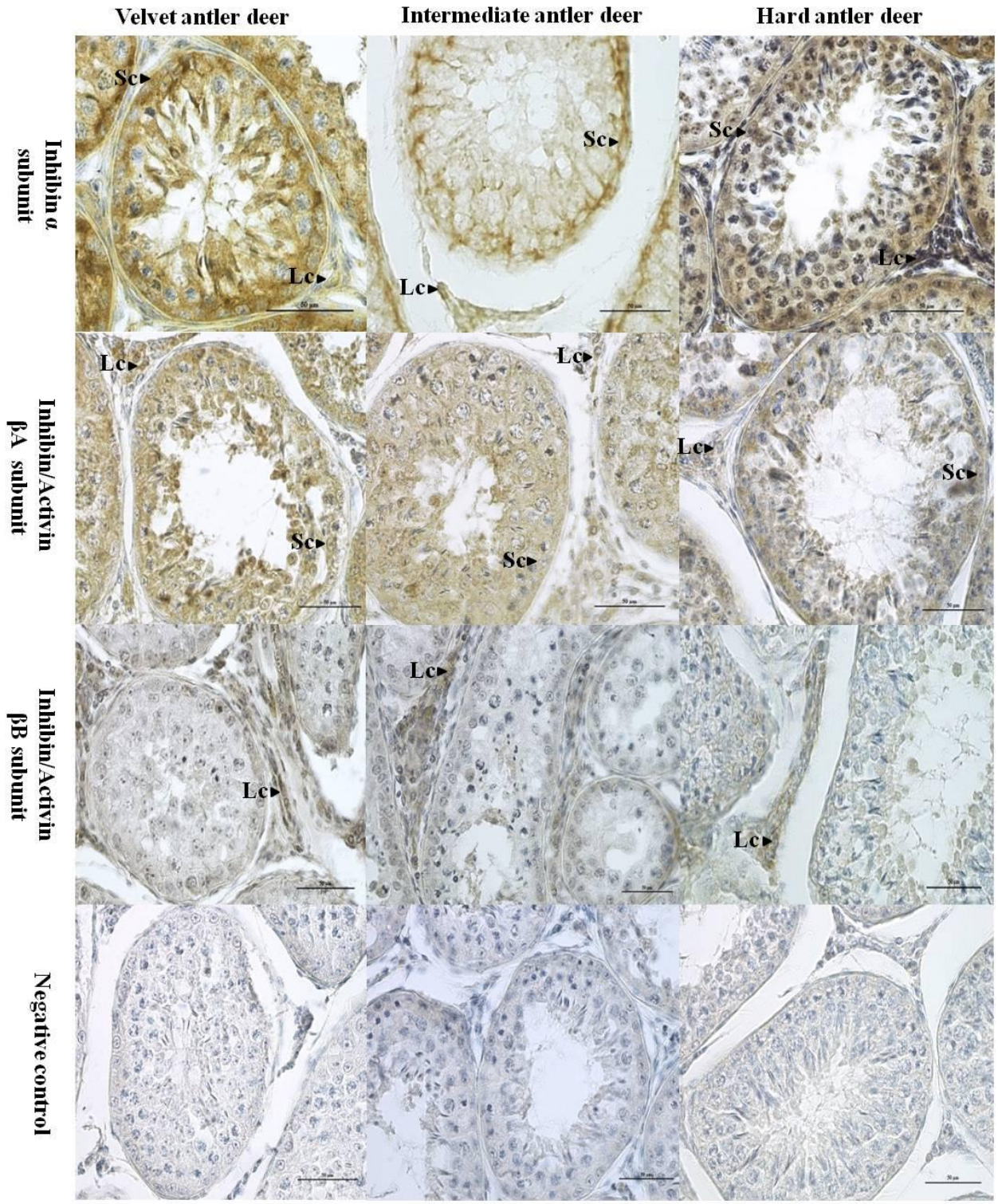


Figure 4 Immunohistochemical localization of inhibin α subunit, inhibin/activin β_A subunit and inhibin/activin β_B subunit in testes of velvet, intermediate and hard antler rusa deer. No immunostaining was detected in control section incubated with normal rabbit serum in place of primary antibody (SCs, Sertoli cells; LCs, Leydig cells; 40× magnification, bars indicate 50 µm)

Discussion

The present study clearly demonstrates that there is a strong functional relationship between antler growth and testicular function in rusa deer. It indicates that this relationship is involved with the level of testicular circulating hormones, such as testosterone and inhibin, during antler growth. From the results, the average testicular weight in the hard antler deer was higher than that of the velvet antler deer. For the histological results, the seminiferous tubule diameter and epididymal spermatozoa were also higher in the hard antler deer than in the velvet antler deer. A previous study reported that complete spermatogenesis could be observed in the breeding season (Suwanpugdee and Moonjit, 2007). This

probably indicates that spermatogenesis and testicular function are at the maximum in the breeding season. Seasonal changes in male reproductive functions, testicular and epididymal physiology and antler cycles have been reported in several deer species including red deer (*Cervus elaphus*) (Gizejewski, 2004; Lincoln, 1971), white-tailed deer (*Odocoileus virginianus*) (Mirarchi et al., 1977), axis deer (*Axis axis*) (Loudon and Curlewis, 1988), fallow deer (*Dama dama*) (Asher and Peterson, 1991), sika deer (*Cervus nippon*) (Suzuki et al., 1992), roe deer (*Capreolus capreolus*) (Blottner et al., 1996; Schön and Blottner, 2009) and southern pudu (*Puda puda*) (Reyes et al., 1997). The seasonal changes with testosterone secretion cause changes in the androgen target organs such as antlers. Antlers are cast and regrown when circulating levels of testosterone are hardly detectable (Lincoln et al., 1970; Lincoln and Kay, 1979).

Moreover, the caput and corpus regions of the epididymis have higher columnar epithelium than the cauda region. The epididymal epithetlial cells vary among species and regions (Marengo, 2008). Apical cells in the caput and corpus are responsible for maintaining the acidic pH of epididymal fluid (Marengo, 2008). Spermatozoa are maturated in the epididymis by epithetlial cells before ejaculation. The cauda region is the major site for storage of spermatozoa, therefore some epithelial cells probably disappear in this region (Robaire and Viger, 1995).

In endocrine regulation of testicular function, Kisspeptin stimulates GnRH secretion within the hypothalamus. Then, GnRH stimulates secretion of luteinizing hormone (LH) and FSH from the anterior pituitary gland that bind to receptors in Leydig and Sertoli cells of the testis, and activate testicular functions such as spermatogenesis and secondary sex characteristics through testosterone and inhibin. Testosterone is produced mainly by Leydig cells of the testes through action of LH (Mclachlan et al., 1996, França et al., 2005). The testosterone hormone is an essential hormone for antler development in addition to stimulation of testicular function (Wislocki et al., 1947; Lincoln, 1973; Goss, 1968; Suzuki et al., 1992; Price et al., 2000; Willard and Randel, 2002; Peles et al., 2003; Schön et al., 2004; Phraluk et al., 2015). Antlers of castrated fallow deer developed conspicuous outgrowths of bone and skin (Kierdorf et al., 2004), but velvet antlers of castrated rusa deer did not fully grow and were dropped (Nikorn Thongtip, personal communication). Increasing circulating testosterone levels prior to the breeding season are associated with the formation of a densely structured antler cortex and intense mineralization of the bone (Muir et al., 1988).

To our knowledge, this is the first study which describes the localization of testicular inhibin subunits in rusa deer. The present results suggest that both Leydig cells and Sertoli cells in the testes are the cellular source of bioactive inhibin. In rusa deer, the antler changes in the distribution of inhibin subunits were accompanied by the changes in testes and epididymis, in testicular weight, seminiferous tubule diameter and epididymal spermatozoa. The positive staining of inhibin α and β_A subunits were observed in both Sertoli and Leydig cells while the positive staining of inhibin β_B subunit was only observed in Leydig cells of all different antler stages. These findings also demonstrate that the rusa deer testes produces inhibin not only in the hard antler stage, but also in the velvet antler stage, although the testicular inhibin subunits positively stained were higher in the hard antler group than in the velvet antler group. To our knowledge, there are three groups of male animals that secrete inhibin. The first group secretes both inhibin A and inhibin B such as horse (Nagata et al., 1998), dog (Taniyama et al., 2001), raccoon dog (Weng et al., 2006[a]), Japanese black bear (Weng et al., 2006[b]) and ground squirrel (Sheng et al., 2008). The second group secretes mainly inhibin A as described in ram (McNeilly et al., 2002) and the third group mainly secretes inhibin B as described in rat (Sharpe et al., 1999), golden hamster (Jin et al., 2001[a]; Kawasu et al., 2003), Göttingen Miniature Pig (Jin et al., 2001[b]) and Japanese monkey (Itoh et al., 2003). The present study suggests that rusa deer belongs to the first group. Moreover, these studies reported that the majority of inhibin B subunit was localized in Leydig cells. These results probably suggest that the cellular source of testicular inhibin B secretion is species-specific (Nagata et al., 1998).

In conclusion, the present study clearly shows that the seasonal change in antler growth in male rusa deer correlates with testicular endocrine function and spermatogenesis. However, there is no direct relationship between antler development and cellular source of testicular inhibin hormone. Further studies should examine antler changes in the quantity of testosterone and inhibin in rusa deer testes and clarify the circulating concentrations of testosterone and inhibin in rusa deer.

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บทคัดย่อ

การศึกษาตำแหน่งของอินฮิบินในอัณฑะด้วยวิธีทางอิมมูโนฮีสโตเคมีและการศึกษาเนื้อเยื่อวิทยาของท่อพักและเก็บเซลล์อสุจิในกวางรูซ่าช่วงที่มีระยะการเจริญของเขาที่แตกต่างกัน

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ปัจจุบันการเลี้ยงกวางรูซ่า (*Rusa timorensis*) ในทางปศุสัตว์ได้รับความนิยมและจำนวนเกษตรกรผู้เลี้ยงกวางรูซ่ามีมากขึ้น การศึกษาระบบสืบพันธุ์กวางรูซ่า นอกจากจะเป็นข้อมูลในการพัฒนาด้านปศุสัตว์ ยังสามารถใช้เป็นข้อมูลพื้นฐานในเทคโนโลยีชีวภาพทางระบบสืบพันธุ์ หรือ Assisted reproductive technologies (ARTs) เพื่อเป็นต้นแบบสำหรับการศึกษาระบบสืบพันธุ์สัตว์ตระกูลกวางที่ใกล้สูญพันธุ์ในประเทศไทย การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาเนื้อเยื่อวิทยา (histology) ของอัณฑะและท่อพักและเก็บเซลล์อสุจิ และศึกษาอิมมูโนฮีสโตเคมี (Immunohistochemistry) ของเนื้อเยื่ออัณฑะกวางรูซ่าในช่วงการพัฒนาของเขากวางที่แตกต่างกัน เก็บตัวอย่างอัณฑะและท่อพักและเก็บเซลล์อสุจิของกวางรูซ่าจำนวน 18 ตัวจากโรงฆ่ากวางในอำเภอกำแพงแสน จังหวัดนครปฐม และแบ่งตัวอย่างที่เก็บได้ออกเป็นสามกลุ่มตามลักษณะของเขากวาง (เขากวางอ่อน เขากวางกึ่งแข็งกึ่งอ่อน และเขากวางแข็ง) จากนั้นศึกษาเนื้อเยื่อวิทยาของอัณฑะและท่อพักและเก็บเซลล์อสุจิ และอิมมูโนฮีสโตเคมีของอัณฑะโดยการย้อมแอนติบอดีต่อฮอร์โมนอินฮิบินทั้งสามหน่วยย่อย (อินฮิบินอัลฟ่า อินฮิบินเบต้าเอ และอินฮิบินเบต้าบี) พบว่า กวางรูซ่าที่มีเขาแข็งมีน้ำหนักอัณฑะและขนาดของท่อนำอสุจิ (76.25±9.80 กรัม และ 256.10±7.28 ไมโครเมตร) มากกว่ากวางรูซ่าที่มีเขาอ่อน (40.86±7.41 กรัม และ 197.25±7.23 ไมโครเมตร) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) นอกจากนี้ ในกวางรูซ่าที่มีเขาแข็งพบเซลล์อสุจิในท่อพักและเก็บเซลล์อสุจิมากกว่าในกวางรูซ่าที่มีเขาอ่อน สำหรับการศึกษาอิมมูโนฮีสโตเคมีโดยการย้อมแอนติบอดีต่อฮอร์โมนอินฮิบิน พบว่าในกวางรูซ่าที่มีเขาอ่อน กวางรูซ่าที่มีเขากึ่งแข็งกึ่งอ่อน และกวางรูซ่าที่มีเขาแข็งมีการติดสีของอินฮิบินอัลฟ่าและอินฮิบินเบต้าเอทั้งในเซลล์เซอโทลิ (Sertoli cell) และเซลล์เลย์ดิก (Leydig cell) ในขณะที่การติดสีของอินฮิบินเบต้าบีพบเฉพาะในเซลล์เลย์ดิกเท่านั้น การศึกษาครั้งนี้บ่งบอกได้ว่า อัณฑะของกวางรูซ่าสามารถผลิตอินฮิบินได้ในทุกช่วงการเจริญของเขากวางที่แตกต่างกัน นอกจากนี้ เขากวางรูซ่ามีความสัมพันธ์กับน้ำหนักอัณฑะ ขนาดของท่อนำเซลล์อสุจิ และปริมาณเซลล์อสุจิในท่อพักและเก็บเซลล์อสุจิ ดังนั้น การใช้ลักษณะของเขากวางเพื่อบ่งชี้การทำงานของอัณฑะในกวางรูซ่าสามารถทำได้

คำสำคัญ: ท่อพักและเก็บเซลล์อสุจิ อิมมูโนพยาธิวิทยา กวางรูซ่า อัณฑะ

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