การเปลี่ยนแปลงตามอายุของอัณฑะอิพิดิไดมิสและอัณฑะจากอิพิดิไดมิสส่วนท้ายในสุนัข

Age-related changes in testis, epididymis and caudal epididymal sperm in dogs

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ชลยุทธ พันธุ์มีเชาวน์ : การเปลี่ยนแปลงตามอายุของอัณฑะ อิพิดิไดมิส และอสุจิจากอิพิดิไดมิสส่วนท้ายในสุนัข (Age-related changes in testis, epididymis and caudal epididymal sperm in dogs) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ศุภวิวัธน์ พงษ์เลาหพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ศยามณ ศรีสุวัฒนาสกุล, 67 หน้า.

การศึกษานี้มีจุดประสงค์เพื่อศึกษาผลของอายุต่อคุณภาพตัวอสุจิ การเสื่อมของอัณฑะ การเกิดพังผืดในเนื้อเยื่อของอัณฑะและการเปลี่ยนแปลงการแสดงออกของตัวรับฮอร์โมนแอนโดรเจนและการงอกขยายของเซลล์เนื้อเยื่ออัณฑะโดยใช้โปรตีนเคไอหกสิบเจ็ดเป็นตัวบ่งชี้ โดยสุนัขขนาดกลางเพศผู้สุขภาพดีจำนวน 55 ตัวจะถูกแบ่งออกเป็น 4 กลุ่มเริ่มจาก เด็ก (1 – 3 ปี) จำนวน 14 ตัว ผู้ใหญ่ (มากกว่า 3 – 6 ปี) จำนวน 12 ตัว แก่ (มากกว่า 6 – 9 ปี) จำนวน 14 ตัว และชรา (มากกว่า 9 ปีขึ้นไป) จำนวน 15 ตัว อสุจิจากถุงเก็บตัวอสุจิใกล้อัณฑะส่วนหางผ่านการตรวจคุณภาพตัวอสุจิ ตัวอย่างจากเนื้อเยื่ออัณฑะ ถุงเก็บตัวอสุจิใกล้อัณฑะ (ส่วนหัว ลำตัว และหาง) และท่อน้ำอสุจิถูกเก็บเพื่อขั้นตอนต่อไป บนพื้นที่หน้าตัดของอัณฑะ ระดับของความเสื่อมและการเกิดพังผืดจะถูกประเมินเชิงจิตวิสัย จากนั้นตัวอย่างเนื้อเยื่ออัณฑะจะผ่านการย้อมด้วยสีฮีมาทอกซีลินและอีโอซิน และแมสซองไตรโครมเพื่อตรวจวัดความเสื่อมของท่อสร้างอสุจิและสัดส่วนพื้นที่ที่มีการสะสมของเนื้อเยื่อเกี่ยวพันตามลำดับ การให้คะแนนกึ่งเชิงปริมานความรุนแรงของการเสื่อมของท่อสร้างอสุจิและการกำหนดปริมาณของเซลล์ในอัณฑะเพื่อคำนวนหาดัชนีสเปิร์มมาติกและดัชนีเซอร์ทอไลเซลล์จะถูกกระทำ การสะสมของเนื้อเยื่อเกี่ยวพันในอัณฑะจะประเมินโดยโปรแกรมวิเคราะห์ภาพแพทเทิร์นควอนท์และรายงานผลเป็นร้อยละปริมาณการเกิดพังผืด วิธีการอิมมูโนฮิสโตเคมมิสทรีจะถูกนำมาใช้เพื่อศึกษาแสดงออกของตัวรับฮอรโมนแอนโดรเจนและโปรตีนเคไอหกสิบเจ็ดแล้วประเมินด้วยโปรแกรมวิเคราะห์ภาพนิวเคลียร์ควอนท์ ผลการศึกษาพบว่าร้อยละของการเคลื่อนที่ การเคลื่อนที่ไปข้างหน้าและความมีชีวิตของอสุจิในสุนัขกลุ่มผู้ใหญ่ แก่และชราต่ำกว่าสุนัขในกลุ่มเด็กอย่างมีนัยสำคัญทางสถิติ อายุของสุนัขมีความสัมพันธ์เชิงลบต่อการเคลื่อนที่ การเคลื่อนที่ไปข้างหน้าและความมีชีวิตของอสุจิ ความวิการเชิงปฐมภูมิและทุติยภูมิพร้อมด้วยความวิการหลักและรองในสุนัขชราสูงกว่าสุนัขเด็กอย่างมีนัยสำคัญ ความรุนแรงของการเสื่อมของท่อสร้างอสุจิค่อยๆเพิ่มขึ้นตามอายุ ($p<0.05$) มีระดับสูงสุดในกลุ่มสุนัขชรา (4.7±0.2) และต่ำสุดในสุนัขเด็ก (1±0) ทั้งนี้ยังพบความสัมพันธ์เชิงบวกระหว่างอายุกับความรุนแรงของความเสื่อมของท่อสร้างอสุจิ ระดับของความเสื่อมและการเกิดพังผืดบนพื้นที่หน้าตัดของอัณฑะในกลุ่มสุนัขชรามีความสูงกว่ากลุ่มอื่น ($p<0.05$) นอกเหนือจากนั้นในสุนัขชรายังพบร้อยละการเกิดพังผืดบริเวณเนื้อเยื่ออัณฑะ (30.9±2.5) มีค่าสูงกว่าสุนัขกลุ่มอื่นอย่างมีนัยสำคัญ ผลการทดลองยังแสดงให้เห็นถึงความสัมพันธ์เชิงบวกระหว่างอายุกับระดับของความเสื่อมและการเกิดพังผืดบนพื้นที่หน้าตัดของอัณฑะและร้อยละปริมาณการเกิดพังผืดบริเวณเนื้อเยื่ออัณฑะ ดัชนีสเปิร์มมาติกในสุนัขชราอยู่ในระดับต่ำเมื่อเทียบกับกลุ่มอื่นๆ ($p < 0.05$) ในทางกลับกัน กลุ่มสุนัขชรามีค่าดัชนีเซอร์ทอไลเซลล์สูงที่สุดอย่างมีนัยสำคัญทางสถิติ แม้ระดับการแสดงออกของตัวรับฮอร์โมนแอนโดรเจนไม่ต่างกันในแต่ละกลุ่มอายุ แต่ยังพบความสัมพันธ์เชิงบวกระหว่างอายุกับการแสดงออกของตัวรับฮอร์โมนแอนโดรเจนในอัณฑะ ดัชนีเคไอหกสิบเจ็ดมีระดับต่ำในสุนัขชราเมื่อเทียบกับสุนัขเด็ก ความสัมพันธ์เชิงลบถูกพบระหว่างอายุและดัชนีเคไอหกสิบเจ็ด โดยสรุปการศึกษานี้แสดงให้ว่าการชราภาพมีผลต่อคุณภาพตัวอสุจิที่ต่ำลง การลดลงของเซลล์ต้นกำเนิดและการเกิดพังผืดในอัณฑะพร้อมด้วยศักยภาพของเซลล์ต้นกำเนิดภายในท่อและประสิทธิภาพของขั้นตอนการเจริญเต็มที่ขั้นสุดท้ายและการสร้างสเปิร์ม นอกเหนือจากนั้นอายุที่เพิ่มขึ้นยังส่งผลลบต่อการแสดงออกของตัวรับฮอร์โมนแอนโดรเจนและโปรตีนเคไอหกสิบเจ็ดในเนื้อเยื่ออัณฑะซึ่งอาจมีผลต่อประสิทธิภาพของการสร้างอสุจิโดยเฉพาะในสุนัขอายุมากกว่า 9 ปี

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สาขาวิชา	วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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CHOLAYUTH BHANMEECHAO: Age-related changes in testis, epididymis and caudal epididymal sperm in dogs. ADVISOR: ASST. PROF. DR. SUPPAWIWAT PONGLOWHAPAN, CO-ADVISOR: ASST. PROF. DR. SAYAMON SRISUWATANASAGUL, 67 pp.

The present study aimed to investigate the effect of aging on sperm quality, testicular degeneration, interstitial fibrosis of dog's testis and alteration of androgen receptor (AR) and proliferation using Ki-67 as a marker in testicular tissues. Fifty-five healthy medium-sized dogs were divided into 4 groups; young (1-3 y/o, n=14), adult (>3-6 y/o, n=12), old (>6-9 y/o, n=14) and senile (>9 y/o, n=15). Spermatozoa were flushed from epididymal tails for routine sperm evaluation. Testes, epididymides (head, body and tail) and vas deferens were collected. The degrees of testicular degeneration and fibrosis on cut surface area were subjectively evaluated. Later, collected tissue sections were stained with H&E and Masson-Trichrome staining for evaluation of testicular degeneration in the seminiferous tubule and the area proportion of fibrotic tissue accumulation in testis, respectively. Microscopically, the semi-quantitative severity scoring of seminiferous tubule degeneration and quantification of testicular cells for Spermatic index (SI) and Sertoli cell index (SEI) were performed. Accumulation of the connective tissue in testis was determined using image analysis software (PatternQuant, 3DHISTECH) and quantified as percent of fibrosis. Expression of AR and Ki-67 protein was investigated by immunohistochemistry and evaluated using image analysis software (NuclearQuant, 3DHISTECH). The results showed that significant lower percentages of sperm motility, progressive motility and viability were found in adult, old and senile dogs, compared to young dogs. Animal's age negatively correlated with sperm motility, progressive motility and sperm viability. The primary, secondary, major and minor sperm defects were significantly higher in senile compared to young dogs. Severity of seminiferous tubule degeneration gradually increased with age ($p<0.05$), being highest in senile dogs (4.7±0.2); and lowest in young dogs (1±0). Age positively correlated with the severity of seminiferous tubule degeneration. The score of testicular degeneration and fibrosis on cut surface area of testis was higher in senile than other age groups ($p<0.05$). In addition, the percent of fibrosis was found to be higher in senile dogs (30.9±2.5) compared to other groups. Significant positive correlations between age and degrees of testicular degeneration and fibrosis on cut surface as well as age and the percent of fibrosis were also observed. In senile dogs, SI was the lowest when compared to other groups ($p < 0.05$). Conversely, senile dogs appeared to have the statistically significant highest SEI. Expression levels of AR did not differ among different age groups. However, a positive correlation was found between age and AR expression in the testis. The Ki-67 index was lower in senile dogs compared to young dogs. Negative correlations were found between age and Ki-67 index. In conclusion, the present study demonstrated that senescence was associated with poor sperm quality, germ cell depletion and interstitial fibrosis of the testis as well as tubular germ cell potential and the efficacy of the final maturation process and spermatogenesis. Nevertheless, aging also has a negative effect on AR and Ki-67 protein expression in testicular tissues which may affect the efficiency of spermatogenesis especially in dogs over 9 years old.

Department:	Obstetrics Gynaecology and Reproduction	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Theriogenology	Co-Advisor's Signature	.....
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%	percent
℃	degree Celsius
µg	micrograms
ANOVA	Analysis of Variance
cm^3	Cubic decimeter
DNA	deoxyribonucleic acid
G	Gap phase
G1	Gap 1 phase
G2	Gap 2 phase
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HPG	Hypothalamo-pituitary-gonadal
kg	Kilograms
LH	luteinizing hormone
M	molar
M	Mitotic phase
mL	Milliliters
mM	Millimolar
mm	Millimeters
PBS	Phosphate-buffered saline
S	Synthesis phase
SEM	standard error of mean
v	volume
W	Watt
w	Weight

CHAPTER I

INTRODUCTION

Importance and rationale

Role of aging and its effect have been studied widely in the past decades in humans due to the increasing of life expectancy that put forward an interest in the impact of aging on the whole body system (Plas et al., 2000). Aging, a natural physiological process, is associated with alterations of structures and functions (Perheentupa and Huhtaniemi, 2009). Also, in the aspect of reproduction, reproductive capacity declines with aging in both men and women. The role of aging of the human reproductive system is very different between genders. Ovarian function has an end-point at the arrival of menopause approximately at the age of 50 (Plas et al., 2000). However, male reproductive capacity do not undergo an unavoidably clear-cut cessation (Yi-chao et al., 2013) and men ideally can produce offspring throughout their life. Degenerative alterations in the testes come with oldness (Jara et al., 2004). Alterations in the aging men associate with several organs and regulatory systems. Endocrine system changes, especially hypothalamo-pituitary-gonadal (HPG) axis. Many researchers have been studying and focusing on this area to understand the age-related alterations on morphology and regulatory function of the human testis. Apoptosis is one of the most mentioned factor that plays an important role in these alterations (Kimura et al., 2003).

Age related changes have also been studied in laboratory animal, such as rats and cats (Elcock and Schoning, 1984; Wang et al., 1993; Wright et al., 1993; Chen et al., 1994). Rats and mice have been proposed as good animal models for examining male reproductive aging processes (Kidd et al., 2001). Aging in rodents appears to be related to histological changes in the testis regarding sperm quality (Parkening et al., 1988; Tanemura et al., 1993; Wang et al., 1993). Nevertheless, in some other aspects, these male mammals differ from men. Dog is a model for studies of the male reproductive system, especially to investigate the benign prostatic hyperplasia (BPH) development.

Even though there have only been a few studies on the age-related changes on reproductive system (James and Heywood, 1979; Ewing et al., 1985) such as semen parameter, testicular morphology, testicular function and endocrine system (sex-steroid hormones) (Peters et al., 2000a). Breeders who own advance-aged stud dogs extremely want to use their dogs to achieve the maximum benefits. From our knowledge, most of breeders have their own cut-off age for the dogs i.e. some breeders stop using their stud dogs at age of 5 or 7 years old. Some breeders discharge the dog when dogs are unable to mate or unable to produce offspring due to several reasons e.g. infertility or health status. Regarding the canine breeding management, questions have been raised when the stud dogs should not be used for breeding purpose due to a significant decline in male fertility. Previous studies did not provide conclusions about the relationship between age and reproductive capacities or whether there is a critical age threshold where the alterations occur (Kidd et al., 2001). Moreover, results from different studies in humans are controversial and contradictory (Kidd et al., 2001). In the same line, the controversial results have been reported in male mammals (Parkening et al., 1988; Lowseth et al., 1990; Tanemura et al., 1993; Wang et al., 1993; Peters et al., 2000b; Goedken et al., 2008). Reviewers suggested that contradictory results may due to a lack of well-designed studies (i.e. sample size, material and method or parameter) (Stewart and Kim, 2011). In male dogs, the difference in dog breeds is considered as one of the reasons. The overall median years of age of the dog is 11-12 years old (Adams et al., 2010; O'Neill et al., 2013). In comparison to different breeds of dogs, large breeds live shorter than those of small breeds. Age at which dogs are considered to become geriatric in various sizes of dogs are different (Goldston et al., 1989; O'Neill et al., 2013). It is obvious that healthy aging male dogs have no andropause (Goldston et al., 1989). Many hypotheses have been proposed to explain these alterations induced by the process of aging. Unfortunately, the specific mechanisms are still debatable. Previous studies on age-related reproductive alterations in male dogs may not enough to conclude the trends of aging and there are still many aspects on aged related changes to be examined for better understanding on fertility decline in aging male dog.

CHAPTER II

LIERATURE REVIEW

Mechanism of Aging

Aging is the result of the decline of the adaptive capacity of an organism to its environment. Its multiple causes are all continuous, permanent and unfavorable. Many studies have been done based on the hypotheses to find the underlying causes of aging but there is no agreement regarding any hypothesis. Many articles have noted some of the aging theories, which comprise the telomere theory (Klapper et al., 2001), the specific gene theory (Guarente and Kenyon, 2000), gene expression alteration due either to mutations (Martin, 1997) or altered DNA methylation (Corbett et al., 1993), the immune theory of aging (Franceschi et al., 2000), and the free radical or oxidative stress theory (Finkel and Holbrook, 2000). The physiological properties of tissues or organism are gradually declined by the aging process. Moreover, with aging, the capacity of cells to replicate themselves decreases and the total number of cell-cycle-arrested cells increases. Cellular senescence or cell aging is the state where cells cannot proliferate themselves permanently and they lost an ability to maintain their homeostatic functions (Muller, 2009; Hornsby, 2010). The number of senescent cells increases with aging in tissues. A genetic program or other entropic processes could be a cause of age-related degeneration (Hayflick, 2007; Salminen and Kaarniranta, 2010).

Age-related alterations in reproductive system

Aging markedly affects morphology and function of testis in men (Peters et al., 2000b). Daily sperm production (Johnson et al., 1984a; Matoska and Talerman, 1989), sperm quality (Schwartz et al., 1983) and serum testosterone level (Zumoff et al., 1982; Bremner et al., 1983; Gray et al., 1991) in aged men are lower than those in young men. Histologically, in advanced age men, a decrease of seminiferous tubules diameter, length and volume (Johnson et al., 1984a; Johnson et al., 1986; Paniagua et al., 1991) were also reported. Furthermore, a thickening of seminiferous tubular

basement membrane, the number of Leydig cells, Sertoli cells, germ cells and other interstitial cells in adult human testes decrease with advanced age (Johnson et al., 1984b; Neaves et al., 1985; Paniagua et al., 1991).

Laboratory animals, like rats and cats, have also being used to study some of the alterations (Elcock and Schoning, 1984; Wang et al., 1993; Wright et al., 1993; Chen et al., 1994). Nevertheless, laboratory animals differ from men, in some other aspects. In rodents, we found an unchanged (Wang et al., 1993; Chen et al., 1994), or even increase in number of leydig cells with age in rats (Ichihara et al., 1993). In brown Norway rats, several studies noted that the percentage of normal seminiferous tubules is gradually decrease with age (Wright et al., 1993; Levy et al., 1999), Decreases in sperm concentration and sperm motility were also noted in senile hamster (Calvo et al., 1999)., total sperm count (Wang et al., 1993; Wright et al., 1993).

Nevertheless, in the brown Norway rat, they reported a spermatogenesis and steroidogenesis declining with age in the same way as aging men (Neaves et al., 1987; Vermeulen, 1991). A few studies have investigated the effects of aging or senescence on the appearance of the epididymis, structure and function (Robaire et al., 2006). In aging rabbit, spermatozoa absence from the epididymal lumen as well as some structure of epithelium changes were found (Cran and Jones, 1980). During aging in the cat epididymis, there were hyperplasia of the epithelium and cysts within the epithelium, as well as eosinophilic to amphiphilic round cytoplasmic bodies in interstitial cells, and luminal debris (Elcock and Schoning, 1984). In the senile hamster, Calvo et al. reported a tubular diameter declining in the caudal part of epididymidis (Calvo et al., 1999). In brown Norway rats, Aging also had an effect on the increasing of basement membrane and decreasing in number of principal cells per number of basal cells in all parts of the epididymis, while diameter of lumen and height of epithelium were not altered by aging (Robaire et al., 2006). In rat, the luminal diameter, only in the caudal part of epididymidis is significantly reduced during aging, while the height of epithelium increases only in the corpus part of epididymidis (Robaire et al., 2006).

In addition, age-related alterations also be seen in HPG axis. Serum testosterone (T) levels (including free T, bioavailable or weakly bound T) decrease with age in men (Hermann and Berger, 1999; Morley and Perry, 1999). While the decline of testosterone concentration in bloodstream has been well reported, the mechanism of this event is still unclear (Hermann and Berger, 1999; Hermann et al., 2000; Wang and Stocco, 2005). Hermann et al. (2000) suggested that this mechanism in advanced age men may originate from all three levels of the HPG. The theory of impairment of HPG axis in aging men have been studied and proposed by several studied which includes declined GnRH release from hypothalamus, declined testicular response to hCG/LH, negative feedback impairment of angrogen, decreased numbers of Leydig cells, diminished testicular perfusion and decreased T outflow upon stimulation by hCG (Suoranta, 1971; Harman and Tsitouras, 1980; Neaves et al., 1984; Liu et al., 2005; Veldhuis et al., 2007; Veldhuis et al., 2009). Another study mentioned that the hypothalamo-pituitary impairment arise from a amplitude of the LH secretory pulse declining which also found in aged rats (Bonavera et al., 1997) Moreover, it was found that the LH secretion is altered due to a decrease of GnRH outflow from hypothalamus rather than a reduction of pituitary response to GnRH (Bonavera et al., 1998; Gruenewald et al., 2000).

In male dogs, only a few studies have interested in the age-related alterations in canine testis (James and Heywood, 1979; Ewing et al., 1985). Ewing et al. addressed an increasing of total Leydig cell numbers with age (Ewing et al., 1985). In 1990, a group of Beagle dogs were studied by Lowseth et al. that they found a decrease in relative percentage of germ cells, an increase in relative percentage of size of seminiferous tubule lumen and a significant decrease in the absolute volume (cm^3) of Leydig cells with increased age (Lowseth et al., 1990). However, no statistical difference in total testicular weight (left and right) between age group was found (Lowseth et al., 1990) In contrast to James and Heywood's study in 1979, that reported a difference of total testicular weight between old and young dogs (James and Heywood, 1979). In the aspect of hormonal alteration, serum testosterone level was measured, but no age-

related significant difference in concentration of serum testosterone was observed (Berry et al., 1986; Lowseth et al., 1990).

Sperm DNA integrity

Integrity of sperm genome is important and essential to produce a healthy offspring (Shamsi et al., 2011). However, the conventional sperm measurements as sperm concentration, viability, motility and morphology may not be a good indicator to reveal sperm defects (Gardner et al., 2012). To overcome the cons of conventional sperm measurements, better diagnostic fertility test and biomarker has been studied (Shamsi et al., 2011). At the present time, many studies have been interested in male genomic integrity as one of the parameters determining male fertility (Erenpreisa et al., 2003; Sakkas et al., 2003; Chohan et al., 2006). Sperm DNA integrity assays are believed to be better markers to determine the potential of male fertility than the conventional semen parameters (Prinosilova et al., 2012). A variety of sperm chromatin integrity detection methods have been reported. The sperm chromatin structure assay (SCSA) is proposed as the most suitable method (Evenson et al., 2002) and has been applied to animal including dogs (Garcia-Macias et al., 2006; Koderle et al., 2009; Kim et al., 2010) and also humans (Virro et al., 2004; Check et al., 2005). DNA damage could be the result of chromatin packaging, or happen at the time during spermatogenesis (Sailer et al., 1995). It may be due to extrinsic (e.g., testicular hyperthermia, environmental toxins) or intrinsic factors (e.g., protamine deficiency, excess reactive oxygen species (ROS) levels, apoptosis) which can finally bring an abnormal chromatin structure that is not suitable with fertility (Zini and Sigman, 2009). However, how can the chromatin abnormalities or DNA damage arise in human spermatozoa are not precisely understood. Sperm chromatin packaging defects, programmed cell death, and oxidative stress (OS) have been proposed as three main theories (Gardner et al., 2012). Various sperm DNA damage measurement assays have been developed. Acridine orange (AO) assay is a rapid, simple and inexpensive technique utilizes fluorescence microscope (Shamsi et al., 2011). The AO assay assesses the DNA in sperm nucleus susceptibility to denaturation by acidic induction by quantifying the change of AO fluorescence color from green (native DNA) to red (denatured DNA). The AO

intercalates into single-stranded DNA as an aggregate and binds to double-stranded DNA as a monomer. The monomeric AO, which bound to native DNA shows green color, while the aggregated AO on denatured DNA bound to fluoresces red (Gardner et al., 2012). The proportion of red/total number of red + green shows the percentage of DNA fragmentation which referred as a DNA fragmentation index (DFI) (Choi et al., 2011). Whereas it is unarguable that DFI assessment is valuable to investigate the causes of human infertility, there is a lack of information in the diagnosis of age-related alteration in dogs (Garcia-Macias et al., 2006).

Normal cytology of normal canine testis

In mammal testis, there are two distinct functional parts, the interstitial part and the seminiferous tubules part where spermatogenesis originating, while the synthesis of androgen occurs within the interstitial part. Both testicular parts contain various cell types, and the spermatogenesis highly depends on autocrine-paracrine collaboration among these cell types (Wang et al., 2009). The Sertoli cells, which are responsible for supporting the structure for germ cell development (Russell, 1993; Vogl et al., 2000), facilitating germ cell movement and mature germ cell release (Mruk and Cheng, 2004), as well as the blood-testis barrier maintenance and seminiferous tubular fluid secretion (Waites and Gladwell, 1982), are located in the seminiferous tubules while the interstitial area contains the Leydig cells (Saez, 1994), macrophages, perivascular smooth muscle cells, and vascular endothelial cells. In human, the normal testicular cytology has been studied and described thoroughly. In contrast with other species, there are a small amount of reports about normal testicular cytology. A description of the normal testicular cytology and ratio between cells that assess testicular function, which may make the testicular cytology interpretation easier, were described by Santos et al. in 2010 (Santos et al., 2010). Briefly description of testicular cytology in dogs has been listed as below

- *Sertoli cells (SE)*
    - SE: 30 - 35 µm in diameter, round to oval in shape. The round nucleus (22 µm in diameter) with a single, round, prominent

nucleolus. The cytoplasm is poorly defined, abundant, pale, and often micro-vacuolated. Occasionally, they are found with small, multiple blue granules.

- *Spermatogenic cells (S)*

    - Spermatogonia: smaller than SE, 18 to 20 mm in diameter with round in shape. The oval-shaped nucleus with a diameter of 14 mm show a special characteristic which is parachromatin condensation with semilunar shape. Spermatogonia's nucleus is smaller than the nucleus of spermatocytes but larger than that of spermatids. The nucleolus of spermatogonia are not noticeable, and the cytoplasm is scarce and lightly basophilic.

    - Primary spermatocytes: larger than all of the spermatogenic cells, with 22 - 28 mm in diameter. They are round and sometimes appear as multinucleated cells, most frequently admix with spermatids. They have round nucleus (19 mm) with a cord-like chromatin pattern. The cytoplasm varies from modest to moderate and stained light blue.

    - Spermatids: round and multinucleated cells with 14 to 18 mm in diameter. The nucleuses are round in early spermatids phase to oval in late spermatids phase with 8 to 10 mm in diameter. These 2 phases of spermatids can be classified by the elongated and dark nuclei, as the chromatin become more clustered as maturation process continued. In early spermatids, the cytoplasm is scanty and basophilic with small punctate vacuoles. In multinucleated cells, the cytoplasm between nucleuses is constantly hypochromatic. The identification of morphologic alterations during spermiogenesis is given below

(1) Golgi phase - an acrosomal vesicle and a small tail are visible

(2) Cap phase - the sac of acrosome is frequently detectable, descending along the nucleus which round to pear-shaped with increasingly darker chromatin

(3) Acrosomal phase - the nucleus is elongated and chromatin become darker

(4) Maturation phase - the nucleus completely pass through the nuclear condensation process. Dense fibers that line along the axoneme is clearly noticeable.

Mature spermatozoa (Z) has a small (6 mm), oval-shaped, hyaline nucleus and a thin elongated tail.

- *Leydig cells*
    - 14 to 18 mm in diameter and round in shape, with a round, eccentric nucleus with a clumped chromatin pattern and one or two small nucleoli. The cytoplasm is abundant with multiple punctate vacuoles (varies in size between 2 - 6 mm in diameter).

Apart from identification in dog testicular cytology, human related indices quantitative analysis or indexes, like Sertoli cell index (SEI) and sperm index (SI) has been used as a quantify for spermiogenic and spermatogenic activity (Schenck and Schill, 1988), (Papic et al., 1988). The SEI (number of SE per 100 S) is used to estimate the tubular germ cell potential (Foresta and Varotto, 1992) while the SI (number of Z per 100 spermatogenic cells) is used for estimating the efficacy of the final maturation process and spermatogenesis (Papic et al., 1988). The normal value of SEI & in dogs was calculated by Santos et. al. in 2010 that it is 4.2 ± 0.8. and 26.6 ± 3.8, respectively. (Santos et al., 2010)

The epididymidis is an important part that acts various functions in mammal testis (Hamilton, 1975). Morphological, physiological and maturational alterations of spermatozoa occurs in the epididymis (Orgebin-Crist, 1967; Bedford, 1975). The

epididymis in dog can be divided into 3 parts; 1. Head of epididymis (Caput epididymis) 2. Body of epididymis (Corpus epididymis) and Tail of epididymis (Cauda epididymis). Histologically, there are several cell types of epididymal epithelium (e.g., principal cells, apical cells, basal cells and halo cells), some are found only in specific areas (e.g., narrow cells), while others are located throughout the duct (e.g., principal cells). Each cell types have their own function; 1. Principal cells: major absorptive and secretory cells 2. Basal cells; protective cells 3. Halo cells; immune functions and 4. Apical cells; function is unknown. The four main functions of the epididymis along the lumen are spermatozoa transportation, sperm motility development, sperm fertilizing ability development, and the sperm maturation. After maturation process, mature spermatozoa stores in the tail of epididymis (Robaire et al., 2006).

Spermatogenesis and cell proliferation

One of the regulatory mechanisms during normal spermatogenesis in all male mammal species is cell proliferation. Proliferation of germ cells constantly occurs in all stages of spermatogenic cells (Clermont, 1972) which need a highly collaborated mechanisms between the Sertoli cells and the germ cells. Efficiency of spermatogenesis is up to the spermatogonia proliferative activity and germ cells losing during meiosis and spermiogenesis (Steger et al., 1998). Ki-67, a nuclear antigen expressed in all phases (G1, S, G2 and M phases) except G0 of cell division, is essential to cell proliferation (Kayaselçuk et al., 2003). Immunohistochemistry using monoclonal antibodies against Ki-67 has been proposed as an important tool to assess cell proliferation (Wrobel et al., 1996). Recently, the monoclonal antibody MIB-I was designed as a marker to detect proliferation by recognizing the Ki-67 antigen (Cattoretti et al., 1992). Antibodies against Ki-67 have been used in many species for assessing the growth pattern of proliferating neoplasms (Wrobel et al., 1993) and also extensively used for investigating whether normal or defected spermatogenesis in several species except dog (Wrobel et al., 1993; Wrobel et al., 1996). This Ki-67 antigen marker, in the normal and defected testis, promises a quick and clear result in this events during spermatogenesis, such as spermatogonia proliferation or completion of meiotic divisions (Wrobel et al., 1993). Ki-67 detection results in a granular nuclear staining,

while the nucleoli are infrequently stained by immunohistochemistry (Gasparini et al., 1994; Sarli et al., 2002; Papaioannou et al., 2009). Immediately after mitosis, level of Ki-67 protein is at its lowest quantity (du Manoir et al., 1991) and may not be detected by immunohistochemistry (Wrobel et al., 1996) due to its short biological half-life of 1–2 hours (Duchrow et al., 1994). It can be rarely detected in resting cells or G0 phase which can provide a reliable result of growth fraction assessment (Gerdes et al., 1984; du Manoir et al., 1991; Gerdes et al., 1991). While using Proliferating Cell Nuclear Antigen (PCNA), another proliferation marker, the growth fraction is more likely to be overestimated when analyze than using Ki-67, because PCNA involves DNA repairing (Bravo and Macdonald-Bravo, 1987; Toschi and Bravo, 1988) and its presence in early resting cells phase due to its long half-life around 8-20 hours (Scott et al., 1991).

Expression of Androgen receptor

Androgens, steroid hormones that play an important part in male reproductive system via the hypothalamic-pituitary-testis axis by determining the expression of the male phenotype, plus the development of secondary sex characteristics along with the initiation and maintenance of spermatogenesis (Robaire and Hermo, 1988; Sharpe, 1994). Androgens, by signaling through the Androgen receptors (AR) in male reproductive tissues, are essential for maintaining spermatogenesis by playing as a factor regulator and establish the production of a suitable environment for epididymal spermatozoa maturation (Zhou et al., 2002). AR, one of the nuclear receptor superfamily, performs as a ligand-dependent transcription factor that regulate the expression of androgen-responsive genes. Collaboration between Androgens and AR is important for male spermatogenesis and fertility (Wang et al., 2009). AR can be detected in Leydig cells, Sertoli cells, and most peritubular cells, but not in germ cells (Bremner et al., 1994; Suarez-Quian et al., 1999). Also in efferent ducts, AR is strongly expressed in stromal cells and epithelial cells (ciliated and non-ciliated). In epididymis, AR was intensely expressed in all epithelial cells (Sar et al., 1990; Takeda et al., 1990; Bremner et al., 1994; Vornberger et al., 1994). In contrast, AR can be rarely detected or even absent in principal cells but moderately stained in basal cells, stromal smooth muscle cells were strongly stained in male mouse's vas deferens (Zhou et al., 2002).

There is a general agreement that AR can be detected in Sertoli cells, peritubular myoid (PM) cells, Leydig cells and perivascular smooth muscle cells in testis (Wang et al., 2009). However, using immunohistochemistry, the localization of the AR in male germ cells remains contradictory. Some studies reported that AR is visualized in germ cells in different species (Warikoo et al., 1986; Kimura et al., 1993; Vornberger et al., 1994), but others show that there is no AR staining in the germ cells (Bremner et al., 1994; Suarez-Quian et al., 1999). Recent data has suggested that androgen receptors in rodents and men decline with aging (Haji et al., 1980; Morley, 2001). There is still no clear conclusion of how the decline of androgen levels happens with aging. Many studies suggest that aging related with changes at HPG axis but mostly at the gonadal or testicular part (Gooren, 1996). Recently, there is an evidence that aging affects the secretion and bioavailability of testosterone by several mechanism in old age (Handelsman, 1994).

Objectives of Study:

1. To examine the effect of increasing age on reproductive profiles (conventional sperm parameter, sperm DNA integrity and testicular morphology) of male dogs
2. To study the expression of the Ki-67 proliferative marker and Androgen receptor (AR) in normal canine testicular tissues during post-puberty to advanced age

Keywords (Thai): อายุมาก สุนัข อสุจิ การเปลี่ยนแปลงของอัณฑะและอิพิดิไดมิส

Keywords (English): aging, canine, sperm, testicular and epididymal change

Hypothesis of study:

1. Increasing age has negative effects on reproductive profile (conventional sperm parameter, sperm DNA integrity and testicular findings) of male dogs
2. Senescence in male dogs is associated with proliferating cells alteration by using anti-Ki-67 antigen as a marker and androgen receptor (AR) changes

CHAPTER III

MATERIALS AND METHODS

Animals

Fifty five client-owned adult medium-sized male dogs, weighted between 10 to 23 kg (Goldston et al., 1989), of different breeds were included in this study. Dogs were classified by their age into 4 groups; young (1 to 3 years old), adult (> 3 to 6 years old), old (> 6 to 9 years old) and senile (> 9 years old). Age criteria were adapted from a previous study by Ortega-Pacheco and colleagues (Ortega-Pacheco et al., 2006), and in this study, we added another group of senile dogs aged over 9 years old. There were 14, 12, 14 and 15 dogs per age group, respectively. A complete physical examination, hematology, serum chemistry, were also conducted before the operation. Tissue samples including testes, epididymides and vas deferens were collected after castration at Small Animal Teaching Hospital, the Faculty of Veterinary Science, Chulalongkorn University, Thailand. Animals were submitted for castration for reasons other than testicular diseases, such as benign prostatic hyperplasia (BPH), perineal hernia (HPR), balanoposthitis, or unwanted behavior. Tissue samples with any historically or clinically noticeable pathologic lesions were excluded.

Experimental procedures

Tissue samples were kept in 0.9% (w/v) normal saline solution at room temperature and transported to the laboratory immediately. The connective tissues were removed from the testes and epididymides, later they were washed in saline solution and dried off with clean gauze. Each testis was dissected free from epididymis, later testes were prepared for another process. Caudal epididymal sperm were collected immediately for sperm evaluation.

1. *Caudal epididymal sperm collection*

The epididymis was dissected and cleaned from unwanted tissues and blood clots. The caudal epididymides and vas deferens were isolated from the rest of the epididymis by making a cut between the corpus and the caudal part of epididymis as described in previous studies (Schimming et al., 1997; Schimming and Vicentini, 2001). The retrograde flushing technique of the vas deferens and caudal epididymis were carried out by using a syringe filled with 1 mL of pre-warmed tris buffer solution and introduce a blunted 21G needle into the vas deferens, then we perfused tris buffer solution, injected air afterwards, until all the contents were flushed out of the caudal epididymis (Ponglowhapan et al., 2006). Sperm samples from both epididymis of the same dog were pooled. After that, the sample were collected in a warm plastic tube and evaluated.

2. *Sperm evaluation*

a. Sperm motility, progressive motility and viability

For sperm motility and progressive motility evaluation, 5 µL of the collected sperm sample was dropped on a warmed glass slide (37ºC), covered with a warm cover slip, and then subjectively examined using a phase contrast microscope under 100x magnification. Motility was estimated as the percentage of total motile sperm (0 - 100 %). Progressive motility was scored on a scale from 0 to 5, with 0 for immotile sperm, and 5 for fast and forward moving sperm. For the percentage of sperm viability, 5 µl of the collected sperm sample was mixed with a single drop of eosin-nigrosin stain on glass slide, air dried and evaluated directly. Two-hundred sperm were counted and evaluated under 1000x magnification. Unstained sperm (white) were classified as live and pink-stained sperm were classified as dead. The percentage of alive or dead sperm (0 - 100 %) were recorded.

b. Sperm morphology

Routine sperm evaluation was performed to examine sperm head and tail morphology as described by Johnston et al. (Johnston et al., 2001). For Mid-piece and tail abnormalities of sperm, collected sperm sample was diluted with formal

saline (ratio 1: 20), 200 sperms were counted under phase - contrast microscope with 400x magnification and recorded as percentage (0 - 100 %). For sperm head abnormalities of sperm, a drop of sample was stained with carbol fuchsin-eosin (William's stain), 500 sperm were counted using bright-field microscope with 1000x magnification under oil immersion and recorded the abnormalities as percentage (0 - 100 %). Then, abnormalities of sperm head and tail morphology were categorized as primary defects, secondary defects, major defects or minor defects which adapted from a study by Blom (Table 1) (Blom, 1973).

c. DNA integrity

Sperm chromatin stability evaluation was carried out with the SCSA technique using Acridine Orange staining (AO; Poly sciences Inc., Warrington, PA). AO presents in green color when binding to native DNA helix, and in red color when bond with denatured/fragmented DNA (Darzynkiewicz et al., 1975; Evenson et al., 1985). Collected sperm samples were gently smeared on a glass slide, and air-dried. The slide was fixed in a methanol–glacial acetic acid (Carnoy's solution; 3:1, v:v) overnight at room temperature. Then, the slide was removed from the fixative solution, air-dried, and then stained with AO staining solution (10 mg/mL AO diluted in distilled water) (Sigma Chemical Co., St. Louis, MO, USA) (Liu and Baker, 1992) for 5 min. After staining, the slide was rinsed in distilled water and covered with the cover slip. Two-hundred spermatozoa were evaluated immediately under an epifluorescent microscope. The AO staining solution was prepared by adding 10 mL of 10 mg/mL AO in distilled water to 40 mL of 0.1 M citric acid (Merck, Darmstadt, Germany) and 2.5 mL of 0.3 M Na2HPO4_7H2O (Merck), pH 2.5. The AO staining solution was prepared daily and stored in the dark at room temperature until used. DNA integrity will be evaluated under an epifluorescent microscope (Laborlux-11 Leitz, Jena, Germany) as a function of the fluorescence of the spermatozoa (wavelength of fluorescence = 520 nm) (Bencharif et al., 2008). Two-hundred cells of each sample were counted. The rate of denaturation was determined by DNA fragmentation index or DFI (Choi et al., 2011).

Table 1 *Sperm morphology defects categorized as primary, secondary defects as well as major, minor defects (Blom, 1973)*

3. Gross and histopathological evaluation

a. Macroscopic findings

The testes were sliced in sagittal plane, and cut surfaces were observed for pathological changes. In normal testis, cut surface is bulgy, greyish and firm without evidence of pathological changes while abnormal testes are severe fibrosis and degeneration with flabby to soft consistency. We classified the lesions, using classification adapted from a previously published study (Teankum et al., 2013). There were 4 subclasses: normal, mild atrophy, moderate atrophy and severe atrophy (Table 2).

Table 2 *Classification of pathological changes of macroscopic lesions on cut surfaces of testes. (Teankum et al., 2013)*

Groups	*Normal*	*Mild atrophy*	*Moderate atrophy*	*Severe atrophy*
The degrees of testicular degeneration and fibrosis	Undetectable white streak with bulgy, greyish, firm consistency without evidence of pathological changes	A small white streak with focal area of compressed white to grey areas (>0-25%)	few areas of white streak and compressed white areas (>25-50%)	Prominent multifocal of white streak with flabby and soft consistency (>50%)

b. Microscopic findings

i. Section preparation

Later, each testis and epididymis was cut in wedge shape approximately 1 x 1 x 0.5 cm^3. for microscopic examination with hematoxylin and eosin (H&E) staining (histologic examination) and Masson-trichrome staining (connective tissue deposition). All specimens were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 48-72 hours, then stored in 70% ethanol, embedded in paraffin wax and sectioned at 4 μm thick on non-coated glass slides for H&E and Masson-trichrome staining. On the other hand, gelatin coated glass slides were used for immunohistochemistry. Each tissue sections were stained with H&E or Masson-

trichrome staining, according to standard histological techniques. The appearance of the testes and epididymis was recorded. After the process of staining and immunohistochemistry, sections were scanned by whole-slide digitalization (Pannoramic Scan, 3DHISTECH) for image analysis.

ii. Assessment of Testes

Testicular severity grading

Histological examination was performed and semi-quantitative severity grading was evaluated as previously published (Rehm, 2000; Lanning et al., 2002). This semi-quantitative scoring system categorized severity as described in Table 3. The absence of any finding was included (score 0).

1. Hypospermatogenesis: The absence of some or all of the germ cells within individual tubules. They often contain some debris, giant cells, and/or swollen spermatocytes in the lumen.

2. Tubules with atrophy/hypoplasia: They are often lined only by elongate Sertoli cells with round nuclei, smaller in diameter with smaller lumen than normal tubules, contain no luminal content without any evidence of interstitial inflammation or basement membrane thickening.

Table 3 *The semi-quantitative scoring system for severity grading (Rehm, 2000; Lanning et al., 2002)*

SCORE	CATEGORIZED SEVERITY (% tubules affected)
1 (Minimal)	< 5%
2 (Slight)	5-25%
3 (Moderate)	25-50%
4 (Marked)	50-75%
5 (Severe)	> 75%

* Score 0 representing absence of the finding

Degree of fibrosis

With Masson-trichrome staining, the degree of fibrosis was evaluated by image analysis software, PatternQuant (3DHISTECH) to detect the connective tissue/collagen (blue-stained) which differentiate from red-stained muscle. The blue (collagen) and red (muscle) stained area of the testis, epididymis, vas deferens and testicular capsules were measured. Then, the proportion of collagen and muscle was calculated, producing a percentage area for collagen and muscle in a tissue section and quantified as percent of fibrosis.

Quantification of testicular cells; Spermatic index (SI) and Sertoli cell index (SEI)

The cells in testes of each sections were classified as spermatogenic cells (S), spermatozoa (Z), Sertoli cells (SE), and Leydig cells according to previous described criteria (Papic et al., 1988; Schenck and Schill, 1988; Baker and Lumsden, 2000; Santos et al., 2010). Five-hundred cells were counted per slide in high-power fields (100x) to determine the SI (number of Z per 100 S) in order to estimate the efficacy of the final maturation step and spermatogenesis and the SEI (number of SE per 100 S) in order to estimate the tubular germ cell potential (Papic et al., 1988; Santos et al., 2010).

4. *Immunohistochemistry technique and assessment for Ki-67 protein and androgen receptor*

Later, each testis was randomly cut in wedge shape approximately 1 x 1 x 0.5 cm^3. Each epididymis, after epidydimal sperm collection, and vas deferens were dissected. All of specimens were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 48 to 72 hours, then stored in 70% ethanol, embedded in paraffin wax and sectioned at 4 μm thick on gelatin coated glass slide for immunohistochemistry.

The Avidin-biotin immunoperoxidase technique (VECTASTAIN Elite ABC Kit, Vector Labs, CA, USA) were used for detecting Ki-67 protein and androgen receptor expression. Paraffin wax was removed from sections by immersion in xylene for 10 min and the sections were rehydrated through a graded alcohol series and finally washed in water.

The expression of cell proliferation using monoclonal antibody against Ki-67 was done using monoclonal mouse anti-Human Ki-67 antigen clone MIB-1 (DAKO, Denmark) as a primary antibody. The sections for Ki- 67 detection were placed in in 10 mM citrate buffer, (pH 6) and heated by microwave oven (800 W, 5 minutes x 1, defrost, 3 minutes x 1, 400 W, 5 minutes, 4 times). Then, sections were washed with PBS 3 times. 3% (v/v) hydrogen peroxide in methanol for 30 min was used to block endogenous peroxidase activity, followed by washing with PBS. For blocking nonspecific binding sites, Normal house serum was applied on the slides for 30 min at room temperature. Sections were incubated at room temperature in moist chamber with the primary antibody, diluted 1:100 for 2 hours then incubated with the second antibody (Anti-mouse IgG, biotinylated (made in horse)), diluted 1:200 for 30 minutes. Later sections were washed with PBS 3 times.

On the other hand, the expression of AR using polyclonal antibody against AR was done using Rabbit polyclonal antibody to AR (Santa Cruz Biotech., TX, USA, clone N-20: sc-816) as a primary antibody the sections for AR detection were placed in 10 mM citrate buffer, (pH 6) and heated by microwave oven (750 W, 5 minutes, 6 times). Then, sections were washed with PBS 3 times. 3% (v/v) hydrogen peroxide in methanol for 30 min was used for endogenous peroxidase activity blocking, followed by washing with PBS. Normal goat serum was applied on the slides for blocking nonspecific binding sites for 30 min at room temperature. Sections were incubated at room temperature in moist chamber with the primary antibody, diluted 1:100 for 2 hours then incubated with the second antibody (Anti-rabbit IgG, biotinylated (made in goat)), diluted 1:200 for 30 minutes. Later sections were washed with PBS 3 times.

A solution of 3,3'- diaminobenzidine (ImmPACT™ DAB peroxidase substrate, Vector Labs, CA, USA) containing 1: 1000 (v/v) hydrogen peroxide, a chromogen, were added to the sections the final reaction visualization. Sections were counterstained with Mayer's hematoxylin and mounted with coverslips by using an aqueous mounting media. A negative control for both AR and Ki-67 were used sections from canine testicular tissue. The primary antibody was excluded and replaced with PBS instead. Sections from pubertal canine testicular tissue was used as a positive control section

for AR while sections from canine intestine tissue was be used as a positive control section for Ki-67

For AR expression, the image analysis software, NuclearQuant (3DHISTECH) was used to calculate intensity of positive immunostaining color and number of nuclei. Slide's label was concealed before the analysis to prevent the bias. Then, the area of interest in each section was randomly selected approximately 25 mm^2, avoid the artifact areas. The results were shown as H-score which is the sum of the percent staining multiplied by an ordinal value corresponding to the intensity level (0=none, 1=weak, 2=moderate and 3=strong). With four intensity levels, the score ranges from 0 (no staining in the sections) to 300 (diffuse strong staining). Meanwhile, the number of positive staining nuclei regardless of the intensity was evaluated for K-67 protein expression. The results were shown as positivity index which is the percentage of immunopositive cells per total number of cells in an entire section.

Statistical analyses

All data were manipulated and statistically analyzed using IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. Descriptive statistics including general means and standard error of mean (SEM) of all the continuous data were calculated. Normal distribution of residuals from the statistical models were tested by the Shapiro-Wilk test. The correlations between age (years) and the dependent variables (percentages of sperm motility, percentages of sperm viability, percentage of sperm defects and sperm DNA integrity) and AR H-score were statistically compared by Pearson's correlation methods and A one-way analysis of variance (ANOVA) with post-hoc Bonferroni test was used to compare the differences of means of the age group for sperm parameters and AR H-score. For the differences of progressive motility (0 to +5) between groups were compared using non-parametric method, Kruskall–Wallis test, then Mann-Whitney test were used to locate differences. and the correlation between age and levels of progressive motility was observed by the Spearman's rank sum test. For the differences of the degrees of testicular

degeneration and fibrosis and severity grades between groups were compared using Kruskall–Wallis test, followed by Mann-Whitney test to locate differences. Percent of fibrosis were analyzed by ANOVA with post-hoc Bonferroni test to compare the differences between group. Pearson's correlation was used to test the correlations between age (years) and Ki-67 expression while ANOVA test was used to analyze the differences of Ki-67 expression among 4 groups. The criterion for statistical significance for these analyses was set at $p < 0.05$.

CHAPTER IV

RESULTS

All fifty-five dogs were healthy and did not undergo any post-operative complication. Each pair of collected testes and epididymides found neither historically nor clinically noticeable pathologic lesions and caudal epididymal sperm could be obtained from all samples (n=55), even in the oldest dogs aged 18 years old. The mean age (years) and weight (kg) of 4 age groups are shown in Table 4.

Table 4 *Mean±SEM and range of age (years) and weight (kg) of all groups (n=55)*

Age group	Age		Weight	
	Mean±SEM	Range	Mean±SEM	Range
Young (n=14)	1.2±0.1	1.0-2.0	18.2±1.2	10.1-24.0
Adult (n=12)	4.3±0.4	3.0-6.0	16.6±1.3	9.8-24.2
Old (n=14)	7.3±0.1	7.0-8.0	15.4±1.7	9.5-24.0
Senile (n=15)	11.4±0.7	9.0-18.0	16.3±1.6	9.0-24.0

1. *Caudal epididymal sperm evaluation*

   a. Sperm motility, progressive motility and viability

Sperm total motility, progressive motility and sperm viability of all dogs observed in this study (n=55) were 77.7±1.6% (ranged 50-90%), 3.5±0.1% (ranged 1-5) and 73.2±1.6% (ranged 43-92%), respectively. There were significant differences in sperm parameters among age groups (Table 5). Young dogs showed significantly higher percentage of motile sperm, sperm with progressive motility and sperm viability compared to other groups ($p < 0.05$). No significant differences in the percentage of motile sperm were observed among adult, old and senile dogs. Both progressive motility and viability were significantly lower in senile dogs than those in young and adult groups ($p < 0.05$). Correlation analysis showed that the animal's age correlated

with sperm motility (r = -0.68, n = 55, p < 0.01), progressive motility (r = -0.62, n = 55, p < 0.01) and sperm viability (r = -0.63, n = 55, p < 0.01) (Figure 1).

Table 5 *Mean±SEM of sperm motility, progressive motility, sperm viability and sperm DNA fragmentation in each age group*

Age group	Sperm motility	Progressive motility	Sperm viability	DNA fragmentation
Young (n=14)	87.8 ± 1.1^{A}	4.3 ± 0.1^{A}	84.2 ± 2.0^{A}	1.6±0.3
Adult (n=12)	77.5 ± 3.3^{B}	3.7 ± 0.1^{B}	73.8 ± 2.4^{B}	2.5±0.8
Old (n=14)	77.1 ± 2.0^{B}	3.1 ± 0.2^{BC}	71.1 ± 2.8^{BC}	3.8±1.6
Senile (n=15)	67.7 ± 2.3^{B}	2.9 ± 0.2^{C}	63.2 ± 2.9^{C}	3.2±1.4

Different superscripts (A, B and C) indicate a significant difference between groups (p < 0.05)

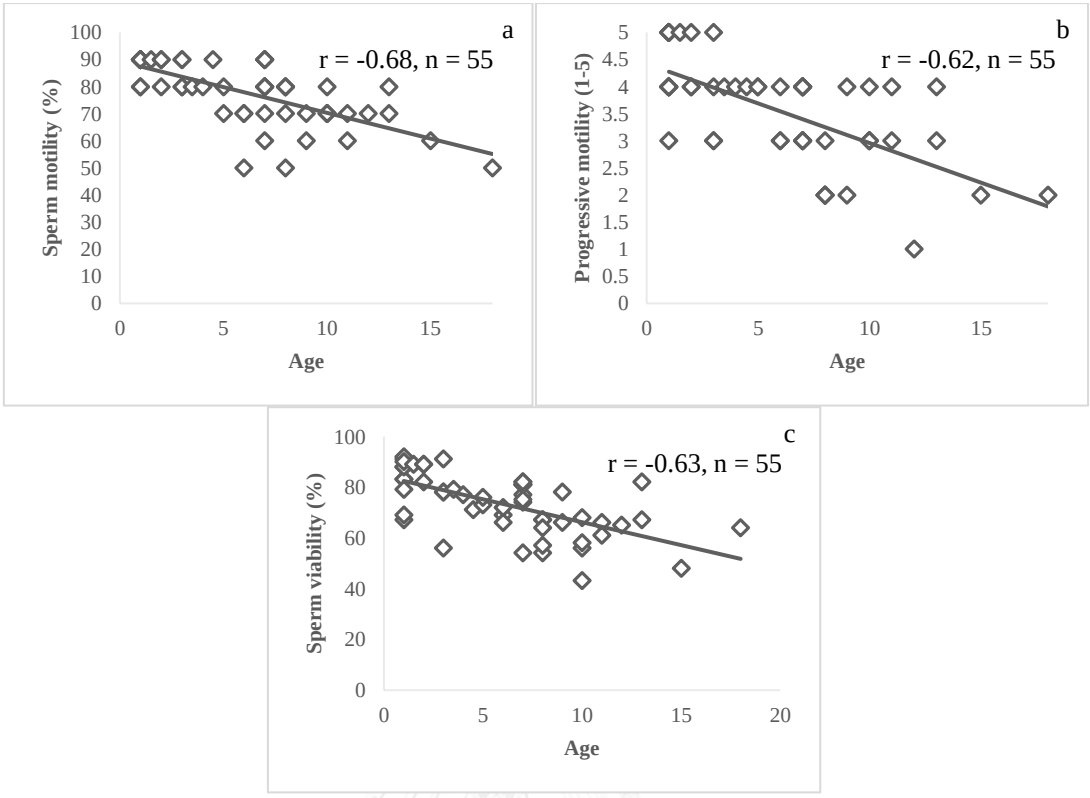

Figure 1 *The correlation between age (years) and percentages of sperm motility (a), progressive motility (b) and percentage of sperm viability (c)*

b. Primary and secondary sperm defects

The proportions of spermatozoa with primary and secondary sperm defects in the study population were 10.8±5.4% (ranged 4.0-27.4%) and 40.5±10.7% (ranged 20.2-75.9%), respectively. Differences in primary and secondary sperm defects were shown among different age groups (Table 6). Higher percentages of spermatozoa having primary and secondary defects were observed in senile group compared to young and adult groups ($p < 0.05$). There were no significant differences in primary and secondary defects when compared between old and adult as well as old and senile dogs. Positive correlations between age sperm defects were observed; primary defects ($r = 0.42$, $n = 55$, $p < 0.01$) and secondary defects ($r = 0.57$, $n = 55$, $p < 0.01$) (Figure 2).

c. Major and minor sperm defects

Of all dogs, the percentage of major and minor sperm defects ranged from 12.4 to 47.7% (25.3±8.4%) and 13.6 to 55.9 (25.9±10.0%), respectively. The age of the dogs had an impact on major and minor sperm defects (Table 6). The percentages of spermatozoa with major and minor defects in senile dogs were significantly higher than young dogs ($p < 0.05$). Major and minor defects did not differ between adult and old dogs. Positive correlations between age and major sperm defects ($r = 0.42$, $n = 55$, $p < 0.01$), and between age and minor sperm defects ($r = 0.48$, $n = 55$, $p < 0.01$) were found (Figure 2).

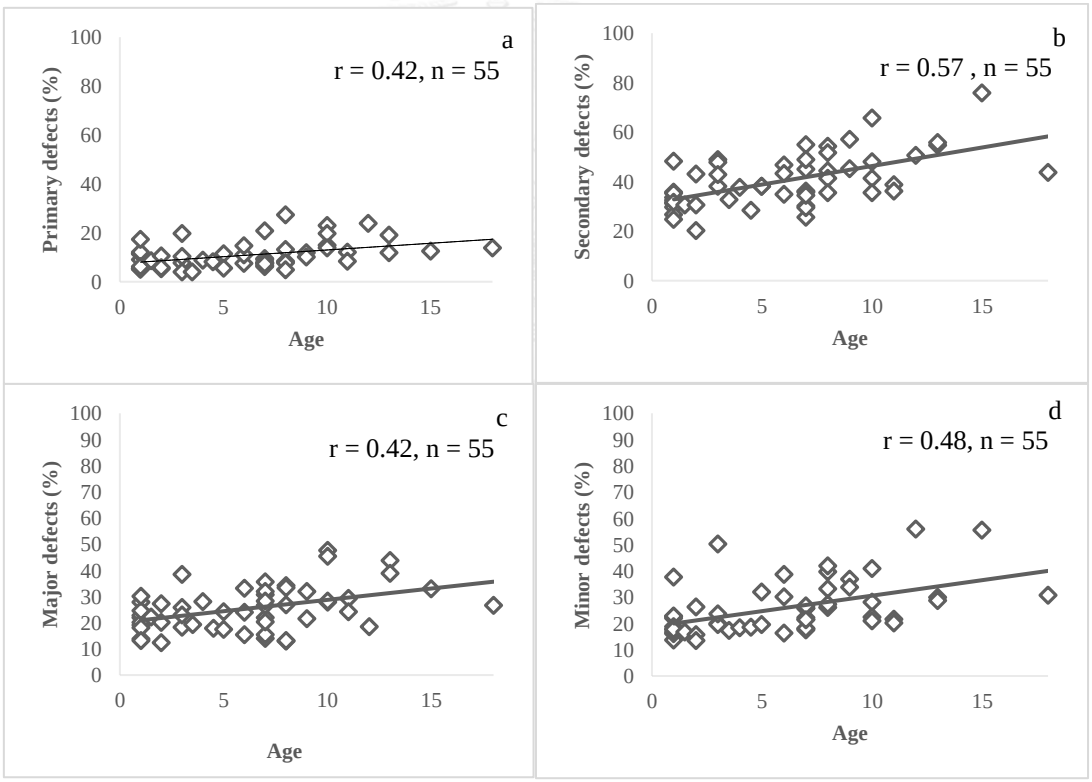

Figure 2 *The correlation between age (years) and percentages of sperm primary (a), secondary (b), major (c) and minor defects (d)*

Table 6 *The percentage (mean±SEM) and 95% confidence interval for mean of primary, secondary, major and minor sperm defects in each age group*

d. Sperm DNA fragmentation

The results showed that the percentage of spermatozoa with fragmented DNA did not significantly differ among age groups ($p > 0.05$). Furthermore, no correlation was observed between age and sperm DNA fragmentation ($p > 0.05$) (Table 5).

2. Gross and Histopathological evaluation

a. Macroscopic findings

Fifty-five pairs of testes were observed. The degrees of degeneration and fibrosis on testicular surfaces ranged from normal (score 1) with bulgy and greyish cut surface to severe fibrosis (score 4) showing flabby and severe accumulation of white streak on cut surface (Figure 3). We found that the degrees of testicular degeneration and fibrosis was higher in senile (3.3±0.2) than other age groups ($p < 0.05$); no difference was found between adult (1.7±0.2) and old dogs (1.8±0.2). (Figure 5) Moreover, Spearman's rank sum test showed the strong significant positive correlation between age and the degrees of testicular degeneration and fibrosis ($r = 0.77$, $n = 55$, $p < 0.01$). (Figure 6)

b. Microscopic findings

i. Assessment of Testes

Testicular severity grading

Histological examination was performed on H&E stained slides of collected testicular tissues. The semi-quantitative scoring system categorized severity as described in Table 2. The score ranged from 1 to 5. The score 0 or absence of any finding could not be detected in any samples (Figure 4). The results showed that severity of germ cell degeneration gradually increased with age ($p < 0.05$), being highest in senile dogs (4.7±0.2; mean±S.E.M) and lowest in young dogs (1±0) (Figure 5). In addition, Spearman's rank sum test showed the strong significant positive correlation between age and the semi quantitative severity grades ($r = 0.93$, $n = 55$, $p < 0.01$). (Figure 6)

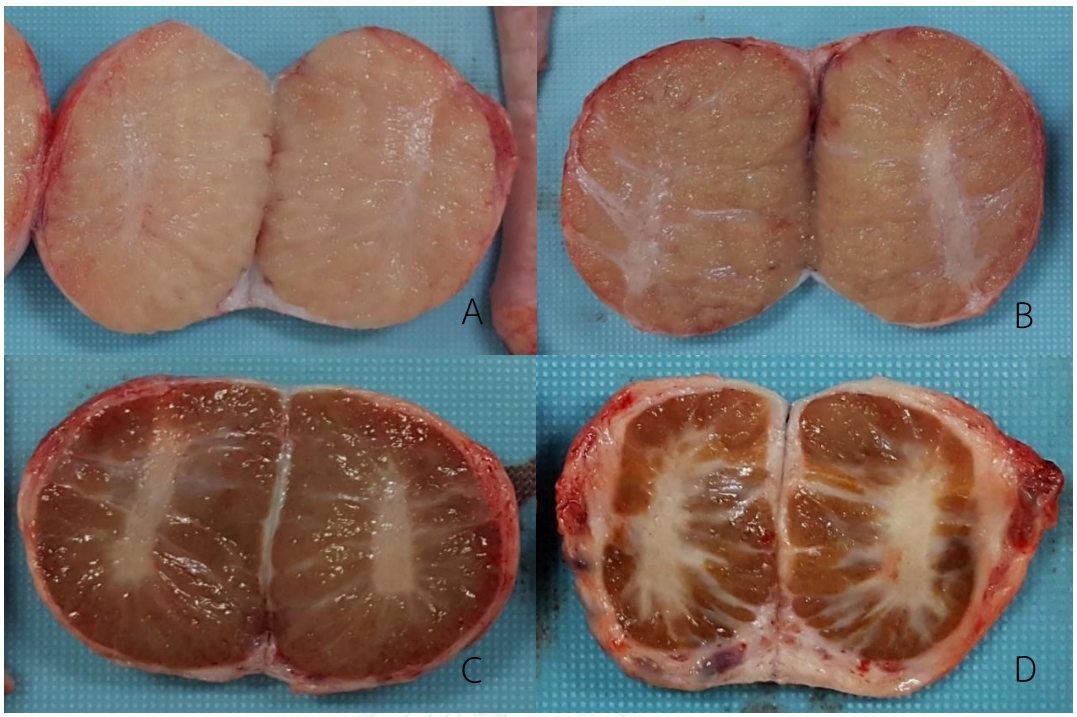

Figure 3 *The degrees of degeneration and fibrosis on testicular surface of collected testes ranged from normal (score 1; A), mild atrophy (score 2; B), moderate atrophy (score 3; C) and severe atrophy (score 4; D)*

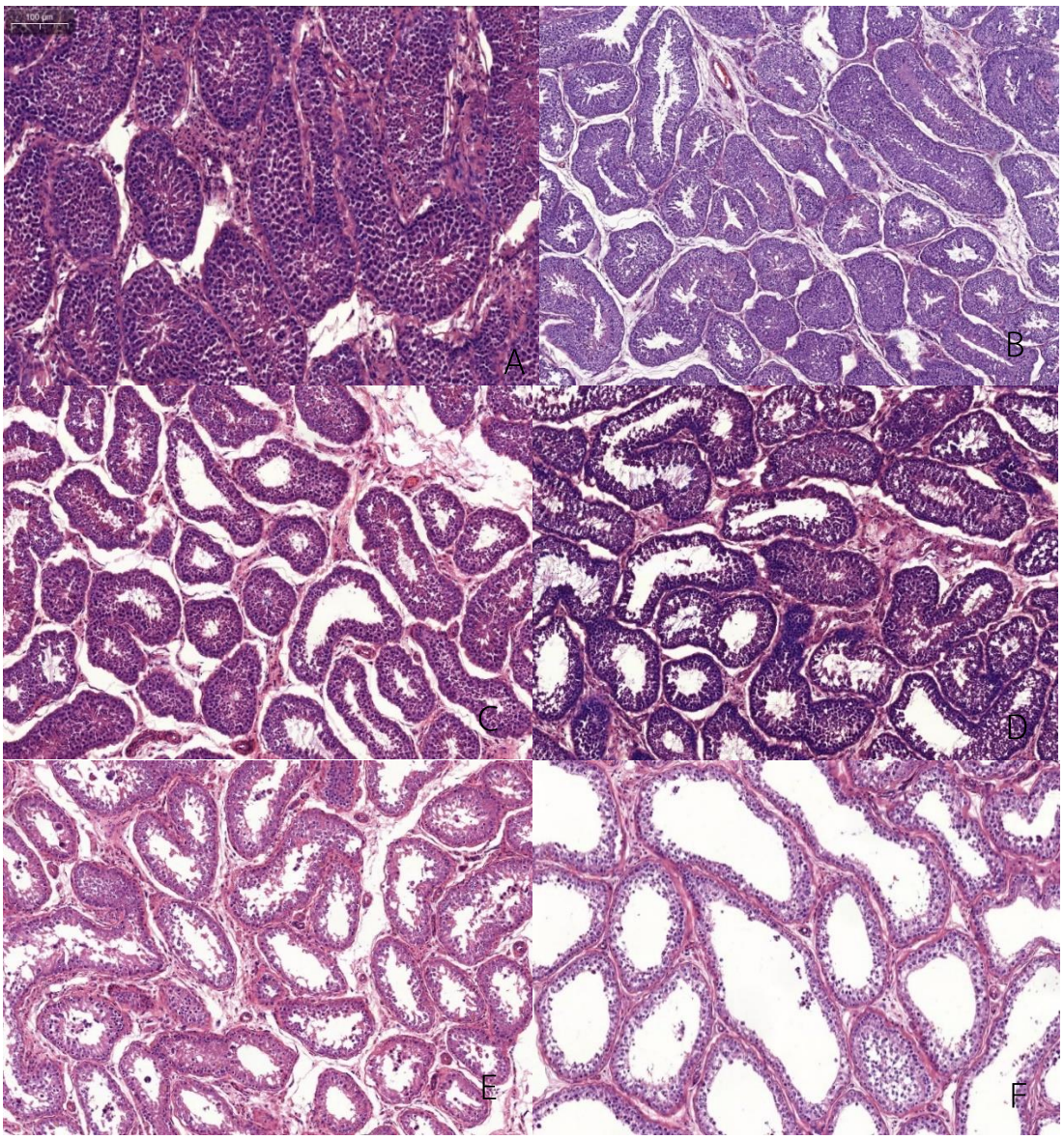

Figure 4 *The semi-quantitative scoring system for severity grading of testicular histology ranged from normal (score 0; A), minimal (score 1; B), slight (score 2; C), moderate (score 3; D), marked (score 4; E) and severe (score 5; F)*

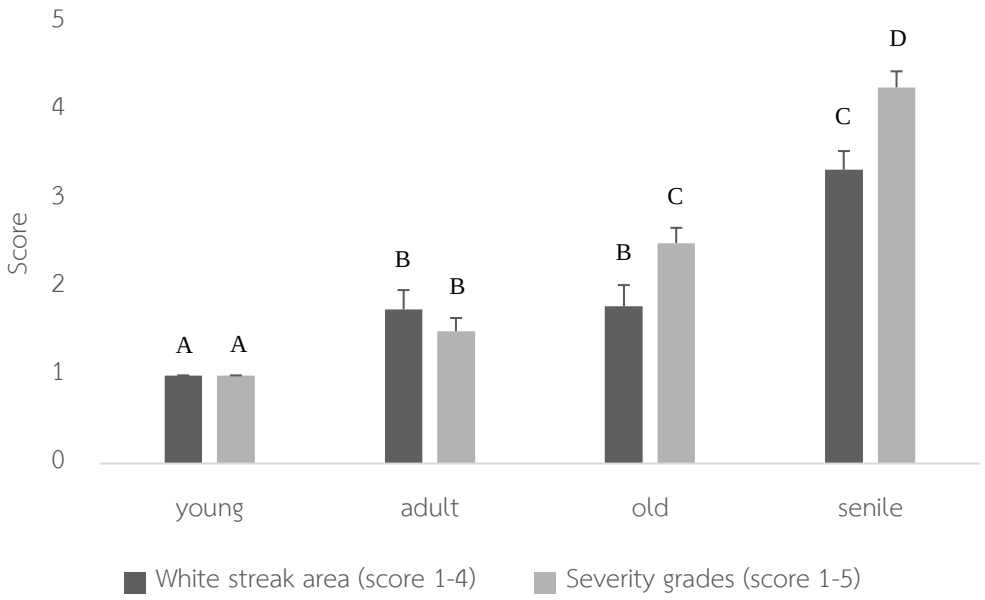

Figure 5 *The degrees of degeneration and fibrosis on testicular surface and the semi quantitative severity grades (severity of germ cell degeneration and atrophy of seminiferous tubules) (mean±SEM) in each age group. Different superscripts indicate a significant difference between groups (p < 0.05)*

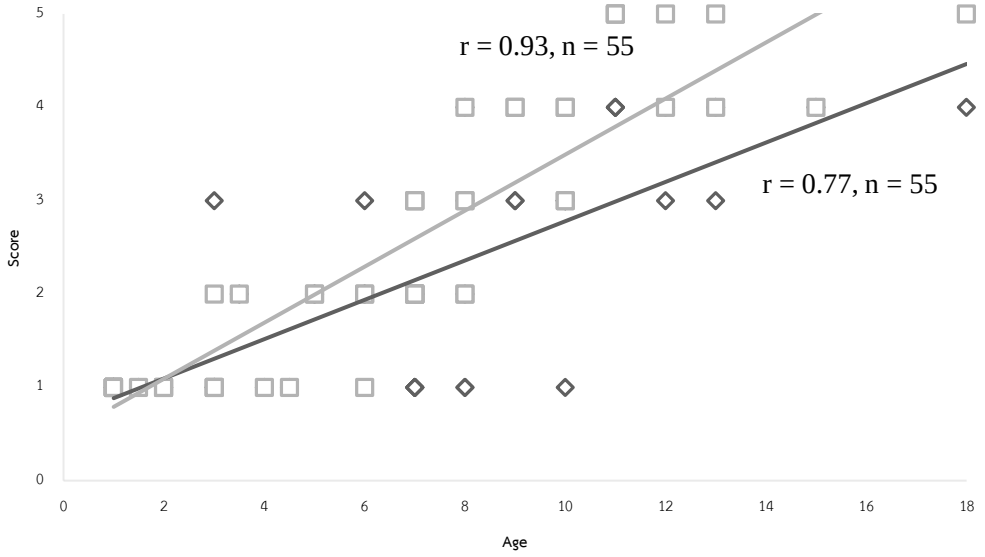

Figure 6 *The correlation between age (years) and the degrees of degeneration and fibrosis on testicular surface (◊) and the semi quantitative severity grades (severity of germ cell degeneration and atrophy of seminiferous tubules) (☐)*

Degree of fibrosis

Examples of the blue-stained connective tissue/collagen and red-stained smooth muscle in the testis, epididymis, vas deferens and testicular capsule are shown in Figure 7. The mean±SEM of percent of collagen in testis, epididymis, testicular capsule and vas deferens were showed in Figure 8. Testicular interstitial fibrosis was found to be higher in senile dogs (30.9±2.5) compared to other groups (young; 13.1±1.3, adult; 17.1±2.1, old; 19.3±2.0, $p < 0.05$). The present of collagen in epididymis was also higher in senile dogs compared to young-adult groups ($p < 0.01$). Meanwhile, the fibrous tissue, in testicular capsule, was significantly lower in young dogs comparing with old and senile groups. In vas deferens, we could not detect any difference of degree of fibrosis among age groups. We also found a positive correlation between age and the percent of collagen in the testis ($r = 0.63$, $n = 55$, $p < 0.01$), epididymis ($r = 0.66$, $n = 55$, $p < 0.01$) and testicular capsule ($r = 0.49$, $n = 55$, $p < 0.01$). (Figure 9)

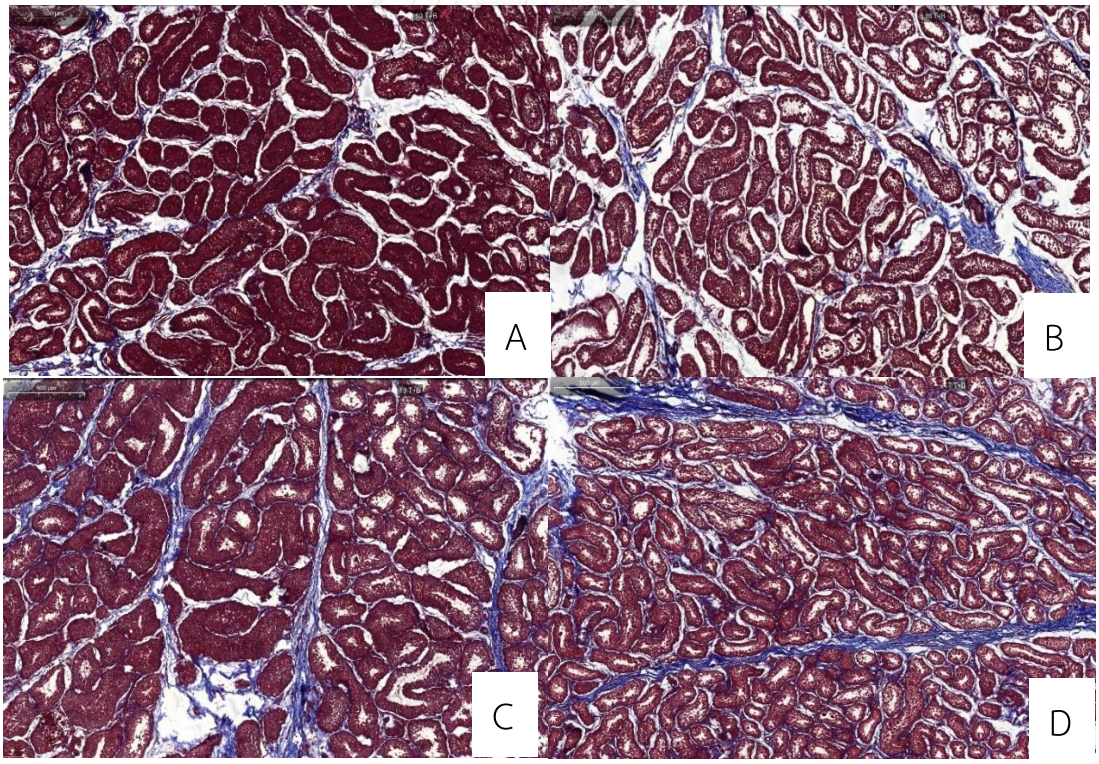

Figure 7 *Examples of the blue-stained connective tissue/collagen in the testis from each age group; young (A), adult (B), old (C) and senile (D)*

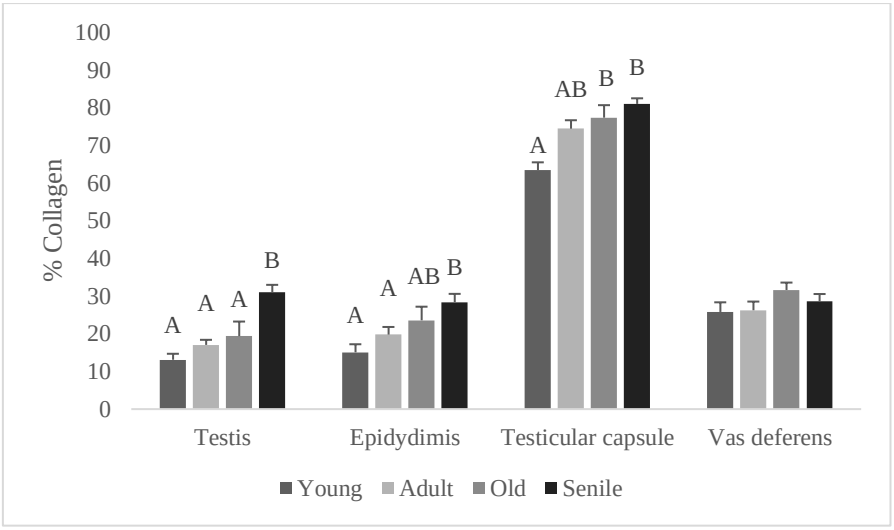

Figure 8 *The Mean±SEM percentage area of collagen (percent of fibrosis) in testis, epididymis, testicular capsule and vas deferens in each age group*

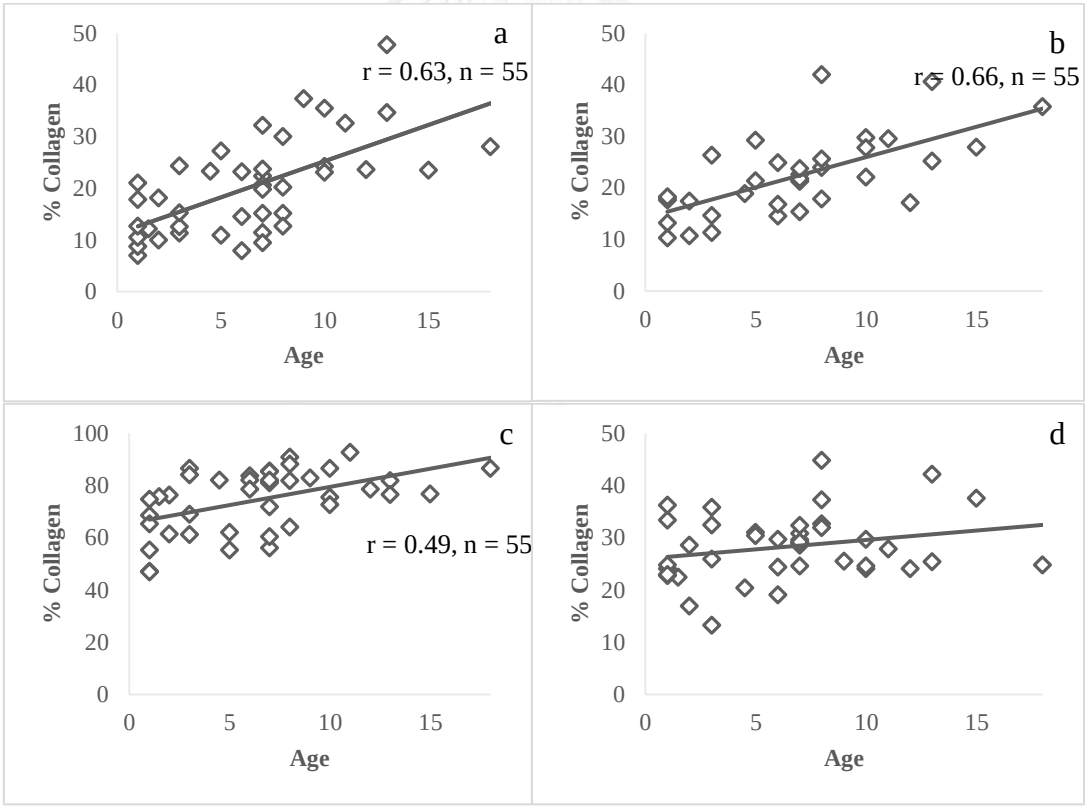

Figure 9 *The correlation between age (years) and the percent of fibrosis in testis (a), epididymis (b) and testicular capsule (c) and vas deferens (d)*

Quantification of testicular cells; Spermatic index (SI) and Sertoli cell index (SEI)

For the quantitative analysis, results were reported in Table 7. All testicular tissue samples and surrounding areas did not show any pathologic inflammatory and/or neoplastic condition. The SI and SEI in this study ranged from 5.8-39.2 and 1.8-15.2, respectively. The results demonstrated the significant differences of SI and SEI among age groups. In senile dogs, SI was the lowest when compared to other groups ($p < 0.05$) while we found no statistical significant differences of SI in young, adult and old groups. Conversely, senile dogs appeared to has the highest SEI and also statistically significant differed from others. Pearson's correlations also revealed a significant negative correlation between age and SI ($r = -0.69$, $n = 55$, $p < 0.01$) while we found a positive correlation between age and SEI ($r = 0.68$, $n = 55$, $p < 0.01$). (Figure 10)

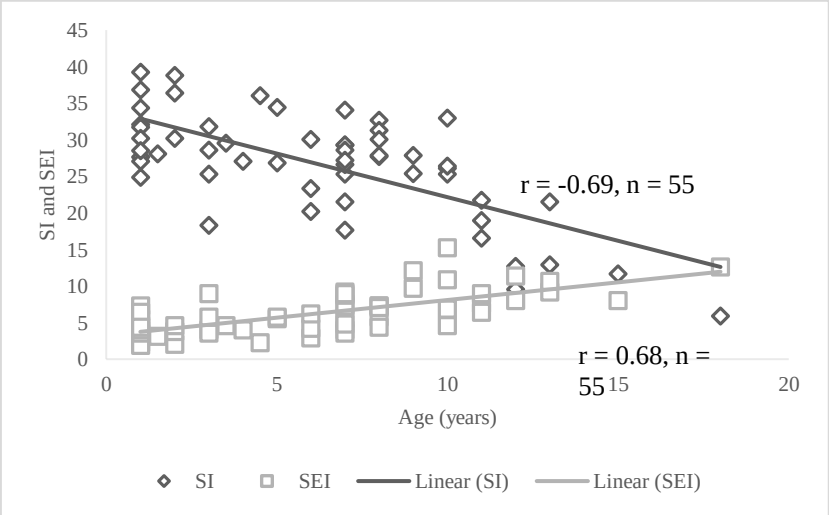

Figure 10 *The correlation between age (years) and SI and SEI*

Table 7 *Mean±SEM of testicular cell types quantification, SI and SEI (range in parentheses)*

3. Immunohistochemistry: Ki-67 protein and androgen receptor expression

a. Androgen receptor expression

The male reproductive tissues, i.e. testis, epididymis (head, body, and tail), and vas deferens used in this study showed positive immunostaining of AR; however, the expression depended on the cell type (Figure 11). Brown-stained positive AR immunostaining was specifically expressed in the nucleus of cells. In testicular tissues, AR was localized in both seminiferous tubules (Sertoli and peritubular myeloid cells) and interstitial portion (Leydig cells), but not in germ cells. The expressions were observed in all epithelial, lamina propria and smooth muscle cells of the epididymis (head, body, and tail) and vas deferens. The results showed no significant differences in the H-score of AR expression in testis, epididymis (head, body, and tail) and vas deferens among age groups (Figure 12). Correlation analysis revealed a positive correlation between age and AR expression in only testicular tissue samples ($r = 0.32$, $n = 55$, $p < 0.05$) (Figure 13). No correlation between testicular AR expression and sperm defects was observed ($p > 0.05$).

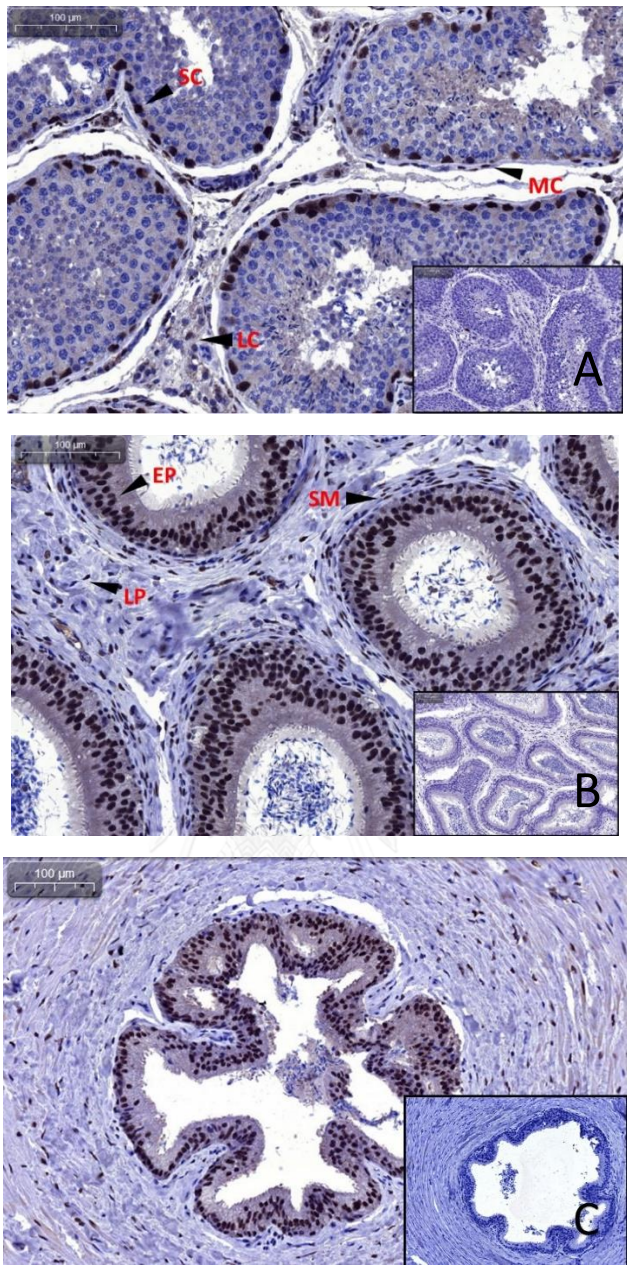

Figure 11 *The positive AR immunostaining in the testis (A), epididymis (B) and vas deferens. The negative controls are shown at the right lower corner. Arrowheads indicate the positive staining cell. In the testis, the AR localization was observed in the nucleus of Sertoli (SC), Leydig (LC) and peritubular myeloid cells (MC) but not in germ cells (A). In the epididymis (B) and vas deferens (C), they were observed in the epithelium (EP), lamina propria (LP) and smooth muscle cells (SM)*

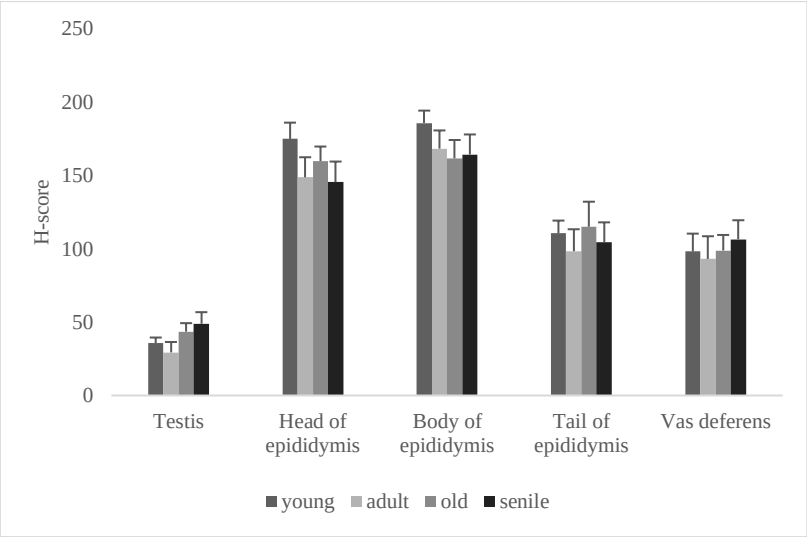

Figure 12 *The androgen receptor H-score (mean±SEM) in testis, each part of epididymis and vas deferens in each age group*

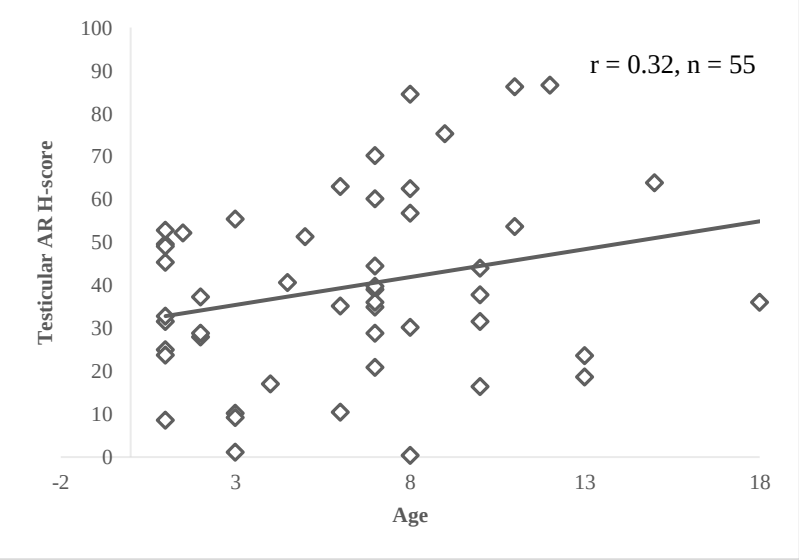

Figure 13 *The correlation between age (years) and Testicular AR H-score*

b. Ki-67 protein expression

In this study, Ki-67 protein expressed mainly in the canine testicular spermatogenic cells (spermatogonia, spermatocytes, and round spermatids) while no positive staining exhibited in the somatic testicular cells (i.e. Sertoli, Leydig cells). (Figure 14) Comparing between two spermatid cells' type, only round spermatid exhibited proliferative activity. Ki-67 protein was intensely expressed in nearly all seminiferous tubules of canine testes in different spermatogenic cells of canine seminiferous tubules at different stages of spermatogenesis.

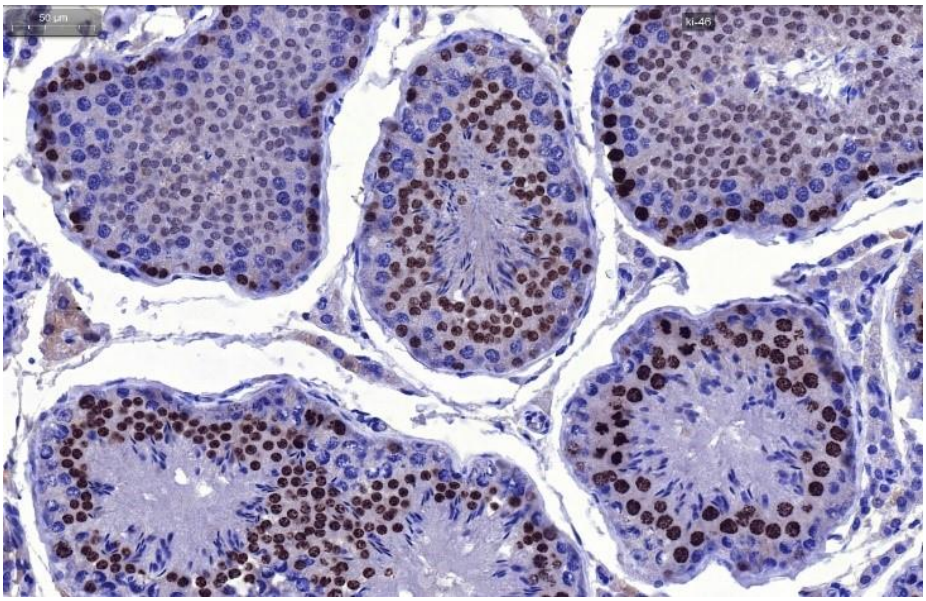

Figure 14 *Positive Ki-67 expression (brown staining) in different spermatogenic cells of canine seminiferous tubules at different stages of spermatogenesis. spermatogonia, spermatocytes and round spermatids (bar= 50 µm)*

The positivity index of Ki-67 expression was significantly lower in senile dogs compared to young dogs (Figure 15). The result in this study also demonstrated a negative correlation between age and Ki-67 immunopositive staining (Figure 16).

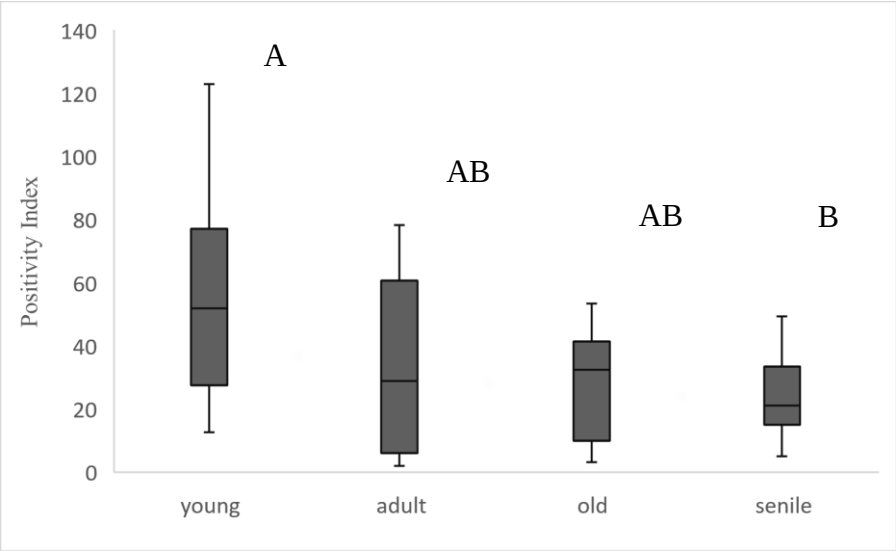

Figure 15 *The box plot shows the positivity index of testicular Ki-67 expression in each age group; young: 56.6±8.2, adult: 36.5±7.4, old: 28.8±4.5 and senile: 24.1±3.5, respectively. Different superscripts (A, B) indicate a significant difference between groups (p < 0.05).*

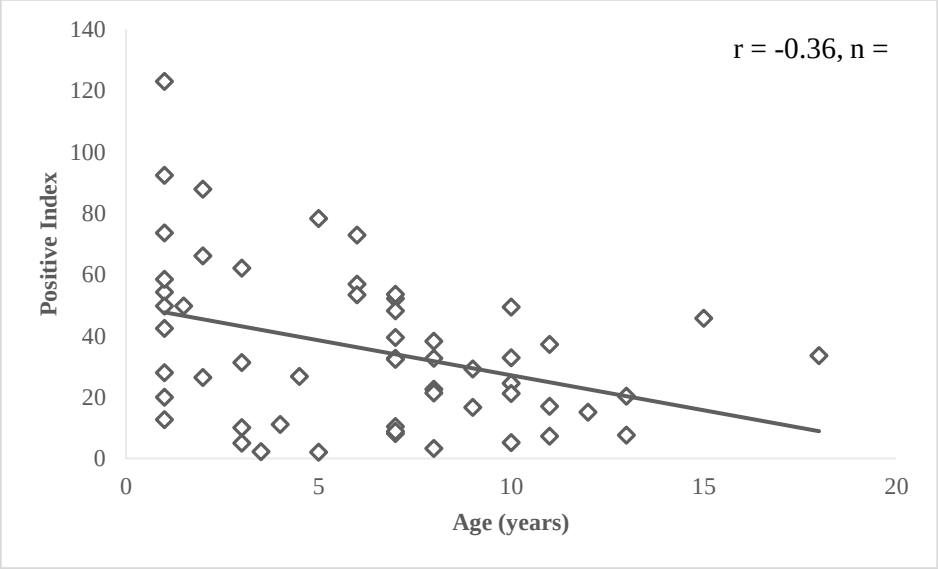

Figure 16 *Correlation coefficient (r) between age (years) and testicular Ki-67 expression in dogs*

CHAPTER V

DISCUSSION

The findings in this study clearly indicated the effect of age on sperm defects from post-puberty to advanced age. All sperm quality parameters observed in this study were affected by increasing age except for DNA fragmentation. The results of our study demonstrated that young dogs (1-3 years old) appeared to have superior quality of sperm compared to other age groups, then the quality started to decline with increasing age regarding to the negative correlation between age and sperm parameters. An age-related increase in the incidence of poor sperm quality potentially compromised the reproductive capacity in male dogs, especially in dogs over 9 years old. Age-dependent changes in sperm qualities have been reported in rat (Wang et al., 1993; Wright et al., 1993), hamster (Calvo et al., 1999), ferret (Wolf et al., 2000), cat (Elcock and Schoning, 1984) and human (Neaves et al., 1987; Vermeulen, 1991). Although our findings were in agreement with results of previous studies demonstrating a significant higher percentage of morphologically abnormal spermatozoa in aging dogs (Rijsselaere et al., 2007; Rota et al., 2016), different sources of sperm samples had to be considered. In this study, spermatozoa were harvested from tail of the epididymis where mature sperm are stored until ejaculation whereas ejaculated sperm were used in previous studies. To avoid an impact of prostatic fluid on sperm morphology, epididymal sperm may serve as a more suitable sperm sample to investigate the age effect on sperm morphology in the dog because incidence of the canine prostatic disorders markedly increases with age. Benign prostatic hyperplasia is strongly correlated with age and is commonly diagnosed in middle to advanced age (> 5 years old) (Atalan et al., 1999). Differences in morphologically normal spermatozoa between ejaculated and epididymal samples have been proved in domestic cats (Axner et al., 1998). A higher proportion of cat sperm with tail abnormalities is obtained from the ejaculates, while a lower proportion of sperm with distal droplets is found in the ejaculates compared to sperm in the cauda epididymides (Axner et al., 1998).

The body size was one of the criteria in this study. Medium-sized dogs were selected because the age at which dogs are considered geriatric are different ranging from 7.5 to 11.5 years depending on body size and breed (Goldston et al., 1989; O'Neill et al., 2013). At the age of 10.9 years, medium dogs are considered geriatric (Goldston et al., 1989). Age of senile dogs in the present study ranged between 9 and 18 years old (11.4±0.7) and sperm defects were more pronounced in this age group. Morphologically, the sperm quality was significantly lower in senile dogs at around the age of 9. Primary and secondary defects as well as major and minor defects of old dogs did not differ from young dogs. Interestingly, the percentages of sperm motility, progressive motility and sperm viability of old dogs (> 6 to 9 years old) remained acceptable and were within normal values of dog sperm quality. Taken together, according to the quality of sperm, male dogs could be used for breeding purposes up to 9 years old. Spermatozoa could be collected from the epididymal tails of all senile dogs, even in an 18 years old dog, with the mean sperm motility of 68%. Although sperm motility and viability was not poor, significant increases in morphologically abnormal sperm may reduce male fertility of senile dogs.

Abnormalities of sperm head and tail morphology were categorized as primary and secondary defects by area of defects while major and minor defects were grouped in association with fertility. Our findings suggested that age affects sperm both during spermatogenesis in the testis and during sperm epididymal storage and translocation.

Many etiologies of sperm DNA damage have been proposed. Fragmented DNA may occur from anomalous chromatin packaging during spermatogenesis, faulty apoptosis, reactive oxygen species (ROS) overproduction, declined seminal antioxidants (Cho et al., 2001; Zhang et al., 2006; Zini and Sigman, 2009; Choi et al., 2011). External factors such as pollution, chemical reagents, diseases, inappropriate testicular temperature and advanced age have also been associated with increased sperm DNA damage (Zini and Sigman, 2009; Shamsi et al., 2011). In humans, an increase in sperm double-stranded DNA breaks with age and sperm chromatin integrity in the advanced age group was markedly damaged (Singh et al., 2003; Paasch et al., 2010). However, results of this study found no influence of age on DNA fragmentation of dog

spermatozoa. It is possible that there is species difference in sperm chromatin integrity regarding the effect of aging or the method used to examine DNA breakage in this study was insufficiently sensitive to detect such differences. There are some other techniques to assess chromatin or DNA damage with pros and cons (Agarwal and Said, 2012).

The overall results from macro- and microscopic findings showed that germ cell proliferation in seminiferous tubules and canine testicular and epididymal interstitial fibrosis affected by the influence of aging. Germ cell proliferation decreased while interstitial fibrosis in both testis and epididymis increased. Atrophy of seminiferous tubules and an increase in connective tissue, both collagen and elastic fiber, in the peritubular space in relation to animal's age has been reported in the testes of horses (Fukuda et al., 2001). The etiologies of fibrosis are still unexplainable. Mechanical, hormonal, immunological, or any combination of these or other factors might cause fibrosis. Any evidence of inflammatory reactions was not being observed in these specimens. Fibrosis is commonly an irreversible change in many organs and in pathological status. Masson-Trichrome staining is suitable to evaluate the testicular and epididymal fibrosis due to blue-stained collagen fibers that differ from other elements. Tubular wall thickness, like peritubular fibrosis, has been used for its ease and accuracy to evaluate the fibrosis (Jarow et al., 1985). Fibrosis may decrease germ cells proliferate in seminiferous tubules. Moreover, it blocks testosterone, which is produced from Leydig cell, to diffuse into seminiferous tubules (Setchell, 1986). Furthermore, it has been reported aging has a positive correlation with the volume of peritubular connective tissue in atrophy seminiferous tubules and in a variety of pathological situations (Volkmann et al., 2011).

Regarding the quantification of cells, SI and SEI, in this study, it was clearly indicated the aging effect on the efficacy of the final maturation step, spermiogenesis and spermatogenesis and from post-puberty to advanced age. Both SI and SEI observed in this study were affected by increasing age. The results of our study demonstrated that the efficacy of the final maturation step, spermiogenesis and spermatogenesis started to decline with increasing age. Then senile dogs (over 9 years

old) appeared to have inferior efficacy of the final maturation step, spermiogenesis and spermatogenesis compared to other age groups. An age-related increase in the incidence of poor efficacy of spermiogenesis and spermatogenesis potentially compromised the reproductive capacity in male dogs, especially in dogs over 9 years old. Furthermore, these results also correlated with a sperm quality mentioned before that age-related increase in the incidence of poor sperm quality, especially in senile dogs. A previous study in adults of various species, including dog, it has been reported that amount of sertoli cells constantly exists throughout all stages of seminiferous tubule epithelium (Foote et al., 1972; Wing and Christensen, 1982) which allows the evaluation of SEI, not only to assist the interpretation of the cytologic results but also can estimates the tubular germ cell potential (Foresta and Varotto, 1992). An increase of the SEI in previous study in human indicates mild or severe hypospermatogenesis (Foresta and Varotto, 1992). To our knowledge, in veterinary cytology, SEI has been evaluated only in horse (Leme and Papa, 2000) and dog (Santos et al., 2010). In dogs, it is widely accepted that an increasing SEI reflects hypospermatogenesis and fibrosis-induced pathologic shortening of the tubules (Dahlbom et al., 1997) which explain the incidence of the age-related increase of SEI in this study. The normal value of SEI has been reported in several species like dogs (SEI = 4.2±0.8) (Santos et al., 2010), stallion (SEI = 20.9±17.0) (Leme and Papa, 2000) and men (SEI = 30±19.5) (Foresta and Varotto, 1992). On the other hand, SI has been used to define the efficacy of the final maturation step and spermiogenesis (Papic et al., 1988). In contrast with SEI, SI progressively decreases in hypospermatogenesis, maturation arrest, and ‘‘Sertoli cell–only’’ syndromes (Foresta and Varotto, 1992) which, in this study, can be described by a negative effect of aging on the tubular germ cell proliferation, represented by Testicular severity grading. The normal SI value has also been studied in dogs (SI = 26.6±3.8), in stallion (SI = 31.5±8.5) and men (SI = 34.8±13.3) (Foresta and Varotto, 1992; Leme and Papa, 2000; Santos et al., 2010). Furthermore, regarding to a study in the normal dog and human testis, no differences in the quality or quantity of spermatogenesis of both testes were observed between bilateral aspirates of testes in a previous histologic study (al-Jitawi et al., 1997; Batra et al., 1999; Peters et al., 2000b; Panikar et al., 2004). Using testicular Fine-needle aspiration technique (FNA) may has

many advantages but there are some limitations like lack of standardization about qualitative and quantitative findings (Leme and Papa, 2000; Santos et al., 2010). On the other hand, the technique we used in this study for slide scanning (whole-slide digitalization) allowed us to explore and quantify the following different testicular cells in wider area and more precise.

There is a general agreement that AR can be detected in Sertoli cells, peritubular myoid (PM) cells, Leydig cells and perivascular smooth muscle cells in the testis (Wang et al., 2009). However, localization of AR immunostaining in male germ cells remains contradictory. Some studies reported that AR is visualized in germ cells in different species (Warikoo et al., 1986; Kimura et al., 1993; Vornberger et al., 1994), but others showed no AR expression in germ cells (Bremner et al., 1994; Suarez-Quian et al., 1999). Our findings showed that AR expressed in all cell types, but not germ cells, of the reproductive tissues of dogs which also been reported in rodents (Takeda et al., 1990). Positive AR immunostaining indicated that the testis and epididymis are target sites of androgen, acting through its receptor, essential to initiate and maintain spermatogenesis in seminiferous tubules (Vornberger et al., 1994) and epidydimal secretory function (Zhu et al., 2000). Earlier studies have established that the epididymis strictly requires testicular androgens for its development, maintenance of tissue homeostasis (Orgebin-Crist, 1967) and epidydimal blood flow regulation (Setchell et al., 1964; Ungefroren et al., 1997). Expression of AR in vas deferens of dogs was in agreement with a study by Dupuy and colleagues (Dupuy et al., 1980) and in mouse's vas deferens (Zhou et al., 2002). AR is believed to involve in regulation of secretory epithelia in vas deferens (Weinbauer et al., 2001).

Although no significant differences in AR expression of the testis, epididymis (head, body and tail) and vas deferens among age groups were observed, a positive correlation between age and testicular AR expression was found. An age-related increase in the amount of AR positive immunostaining cells possibly resulted from lower germ cells with increasing age due to hypospermatogenesis or maturation arrest and sertoli cell hyperplasia or ''Sertoli cell–only'' syndromes in relation to degenerative changes and incomplete spermatogenesis in the seminiferous tubules

(Lowseth et al., 1990). Moreover, the number of Leydig cell and incidence of Leydig cell hyperplasia in the dog increase with age due to the compensation for the lack of steroidogenic potential per cell (Ewing et al., 1985; Lowseth et al., 1990). Furthermore, our results suggested that AR do not associate with increased sperm defects in aged dogs as evidenced by no difference in the level of AR expression in the testis and epididymis among different age groups as well as no correlation between AR expression and sperm defects.

In most seminiferous tubules, only a stage of spermatogenesis showed immunolocalized signal of Ki-67 expression. This result revealed that each stages of spermatogenesis subsequently took place in a specific part of seminiferous tubules. The number of immunolabeled cells in each stage was different regarding to the number of spermatogenic cells in testis. In contrast, Ki-67 positive cells were not detected in the epididymal duct in different age groups. It has been reported that cells are proliferating in the epididymal duct of young rats and the cell proliferation decreased with age (Clermont and Flannery, 1970). The results in this study showed that senescence in male dogs is associated with aged-related Ki-67 protein expression. Differences in the Ki-67 index between young and senile group indicate that degree of testicular cell proliferation tend to decline from the age of 9. The lower Ki-67 index in senile dogs and the negative correlation between age and Ki-67 index suggested that these alterations induce by the process of aging event; however, the specific mechanisms remain to be further investigated. Recent evidence suggests that aging affects reproduction in the male by a progressive and more prominent degenerative changes in the testes (Lowseth et al., 1990) and is considered the most common cause of low fertility in domestic animal species (Domingos and Salomão, 2011). A diminution of spermatogenesis capacity has also been reported for human (Johnson et al., 1984a; Neaves et al., 1984; Paniagua et al., 1991), horse (Johnson et al., 1991) and donkey (Nipken and Wrobel, 1997). However, the exact causes and the beginning of these deteriorations are unexplainable. The loss of germ cell naturally occurs during all 3 steps of spermatogenesis (spermatocytogenesis, meiosis and spermiogenesis) even in the period of maximal seminiferous epithelial activity (Amann, 1970; Johnson et al.,

1981; Paniagua et al., 1987). A study in donkeys suggested that the loss rate seems to increase with age, particularly during the meiotic divisions as well as during spermiogenesis. Germ cell loss is the result of either apoptosis or exfoliation. Our results are in accordance with the observation of more frequent germ cell degeneration and sloughing in aging human (Johnson et al., 1984a; Johnson et al., 1984b) and donkey testes (Nipken and Wrobel, 1997). The increasing age may affect the efficiency of spermatogenesis due to the loss of spermatogonia proliferative activity.

In conclusion, the present study demonstrated that senescence has a negative effect on sperm quality, interstitial fibrosis of testis and epididymis, the tubular germ cell proliferation and also AR and Ki-67 protein expression in testicular tissues. Our findings provide a better understanding of the reproductive profiles in relation to animal's age which could be applied to clinical reproduction and breeding management. This finding may assist in the interpretation and assessing infertility in male dogs.

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APPENDIX

Age group	Young (n=14)	Adult (n=12)	Old (n=14)	Senile (n=15)
Caudal epididymal sperm evaluation				
- Sperm motility	87.8±1.1^{A}	77.5±3.3^{B}	77.1±2.0^{B}	67.7±2.3^{B}
- Progressive motility	4.3±0.1^{A}	3.7±0.1^{B}	3.1±0.2^{BC}	2.9±0.2^{C}
- Sperm viability	84.2±2.0^{A}	73.8±2.4^{B}	71.1±2.8^{BC}	63.2±2.9^{C}
- Sperm morphology				
- Primary defects	8.5±1.0^{A}	9.4±1.3^{A}	10.3±1.7^{AB}	15±1.3^{B}
- Secondary defects	32.4±1.8^{A}	39.8±1.8^{A}	40.5±2.5^{AB}	49.8±3.2^{B}
- Major defects	21±1.4^{A}	25.4±1.9^{AB}	24.7±2.2^{AB}	32.1±2.5^{B}
- Minor defects	20±1.6^{A}	25.4±3.2^{AB}	26.1±2.0^{AB}	32.8±3.3^{B}
- DNA fragmentation	1.6±0.3	2.5±0.8	3.8±1.6	3.2±1.4
Gross and Histopathological evaluation in testis				
- Macroscopic findings				
- The degree of testicular degeneration and fibrosis	1.0±0^{A}	1.7±0.2^{B}	1.8±0.2^{B}	3.3±0.2^{C}
- Microscopic findings				
- The semi quantitative severity grades	1.0±0^{A}	1.5±0.1^{B}	2.5±0.1^{C}	4.7±0.2^{D}
- Percent of fibrosis	13.1±1.3^{A}	17.1±2.1^{A}	19.3±2.0^{A}	30.9±2.5^{B}
- SI	31.8±1.2^{A}	27.6±1.5^{A}	27.7±1.1^{A}	19.6±2.0^{B}
- SEI	4.0±1.6^{A}	4.7±0.5^{AB}	6.5±0.4^{B}	9.4±0.7^{C}
Immunohistochemistry				
- AR	35.7±3.7	29.3±7.1	43.4±5.8	48.8±7.8
- Ki-67	56.6±8.2^{A}	36.5±7.4^{AB}	28.8±4.5^{AB}	24.1±3.5^{B}

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