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RELATIONSHIP BETWEEN INSULIN-LIKE GROWTH FACTOR-1 AND SEMEN QUALITY IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*)

Miss Yuqing Yang

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Science and technology
Common Course
FACULTY OF VETERINARY SCIENCE
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ความสัมพันธ์ระหว่างอินซูลินไลค์โกรทแฟคเตอร์วันและคุณภาพน้ำเชื้อในช้างเอเชีย (อิเลฟัส แม็กซิมัส)

น.ส.ยู่ชิง หยาง

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	RELATIONSHIP BETWEEN INSULIN-LIKE GROWTH FACTOR-1 AND SEMEN QUALITY IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*)
By	Miss Yuqing Yang
Field of Study	Veterinary Science and technology
Thesis Advisor	Professor Dr. KAYWALEE CHATDARONG, D.V.M., M.Sc., Ph.D.
Thesis Co Advisor	Doctor Dr. Taweepoke Angkawanish, D.V.M., Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

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ยู่ชิง หยาง : ความสัมพันธ์ระหว่างอินซูลินไลค์โกรทแฟคเตอร์วันและคุณภาพน้ำเชื้อในช้างเอเชีย (อิเลฟัส แม็กซิมัส). (RELATIONSHIP BETWEEN INSULIN-LIKE GROWTH FACTOR-1 AND SEMEN QUALITY IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*)) อ.ที่ปรึกษาหลัก : เกวลี ฉัตรดรงค์, อ.ที่ปรึกษาร่วม : ดร. ทวีโภค อังควานิช อังควานิช

องค์ความรู้ในปัจจุบันบ่งชี้ว่าฮอร์โมนอินซูลินไลค์โกรทแฟคเตอร์-วัน หรือ ไอจีเอฟ-วัน (insulin-like growth factor-1; IGF-1)สัมพันธ์กับกระบวนการการสร้างฮอร์โมนสเตียรอยด์ และการสร้างอสุจิ จากความสัมพันธ์นี้ จึงอาจนำฮอร์โมนไอจีเอฟ-วันมาประยุกต์ใช้สำหรับการเพิ่มคุณภาพน้ำเชื้อในช้างเอเชีย (*Elephas maximus*) ได้ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างระดับฮอร์โมนไอจีเอฟ-วัน และเทสโทสเตอโรน (testosterone) ในเลือด และตัวแปรต่าง ๆ ในน้ำเชื้อ การศึกษานี้เก็บตัวอย่างเลือดและน้ำเชอจากช้างเอเชียรวม 17 ครั้ง (1– 3 ครั้งต่อเชือก) กระตุ้นการหลั่งน้ำเชื้อด้วยการนวดผ่านทวารหนัก(rectal massage) ตัวอย่างน้ำเชื้อถูกตรวจสอบคุณลักษณะทั่วไป และตัวอย่างเลือดถูกนำไปปั่นแยกส่วนเป็นซีรั่มและทดสอบระดับฮอร์โมนไอจีเอฟวันด้วยชุดทดสอบเอนไซม์ลิงค์อิมมูโนซอร์เบนต์แอสเสย์ (enzyme-linked immunosorbent assay; ELISA) และประเมินหาความเที่ยงตรง (precision) และความแม่นยำ (accuracy)ผ่านการคำนวณค่าสัมประสิทธิ์ความแปรปรวนภายในและระหว่างชุดทดสอบ (intra- and inter-assay coefficient variation; CV) และทดสอบค่าความเป็นเส้นตรง (linearity test) และค่าคืนกลับ (recovery test) ตามลำดับ ระดับฮอร์โมนไอจีเอฟ-วันมีความสัมพันธ์เชิงบวกกับสัดส่วนของสเปอร์มาโตซัว (spermatozoa) ที่มีอะโครโซมสมบูรณ์ (intact acrosome) ($r = 0.53$, $P < 0.05$) และที่มีรูปร่างส่วนหัวปกติ (normal head morphology) ($r = 0.48$, $P < 0.05$) นอกจากนี้ระดับของฮอร์โมนไอจีเอฟ-วันยังมีความสัมพันธ์เชิงบวกกับระดับของฮอร์โมนเทสโทสเตอโรนอีกด้วย($r = 0.73$, $P = 0.004$)ในส่วนของความเที่ยงตรงพบว่าค่าสัมประสิทธิ์ความแปรปรวนภายในชุดทดสอบมีค่ 1.6 – 6.4% และระหว่างชุดทดสอบมีค่า 4.7 – 6.9% ตามลำดับในส่วนของความแม่นยำพบว่าค่าความเป็นเส้นตรงภายใต้การเจือจางเป็นลำดับของตัวอย่างซีรั่มที่ทราบระดับความเข้มข้นมีค่าสูงมาก ($R^2 = 0.99$) ร่วมกับระดับการคืนกลับที่ยอมรับได้ (ค่าเฉลี่ย = 114.7 ± 19%, พิสัย = 100–143%) นอกจากนี้พบว่าค่าคืนกลับเฉลี่ยของตัวอย่างสไปค์ (spiked sample) ของฮอร์โมนไอจีเอฟ-วันมีค่าเท่ากับ 107.2 ± 21%(พิสัย = 80.8–136.9%) โดยสรุปพบว่า ระดับของฮอร์โมนไอจีเอฟ-วันที่เพิ่มสูงขึ้นกับคุณลักษณะที่ดีของน้ำเชื้อซึ่งแสดงถึงความสัมพันธ์ระหว่างระดับฮอร์โมนไอจีเอฟ-วันและฮอร์โมนเทสโทสเตอโรน ซึ่งมีความสำคัญต่อความสมบูรณ์พันธุ์ของช้างเพศผู้นอกจากนี้ผลการศึกษายังบ่งชี้ถึงชุดทดสอบอีไลซ่า ที่ใช้ในการศึกษานี้สามารถใช้ประเมินระดับฮอร์โมนไอจีเอฟ-วันในกระแสเลือดของช้างเอเชียได้ การศึกษาในอนาคตควรมุ่งเน้นการพิสูจน์กลไกทางชีวภาพที่ส่งผลต่อคุณภาพน้ำอสุจิของฮอร์โมนไอจีเอฟ-วันในช้าง

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สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Yuqing Yang : RELATIONSHIP BETWEEN INSULIN-LIKE GROWTH FACTOR-1 AND SEMEN QUALITY IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*). Advisor: Prof. Dr. KAYWALEE CHATDARONG, D.V.M., M.Sc., Ph.D. Co-advisor: Doctor Dr. Taweepoke Angkawanish, D.V.M., Ph.D.

Evidence has been increasingly suggested that insulin-like growth factor-1 (IGF-1) is related to steroidogenesis and spermatogenesis. This may be conducive to find the causes of poor sperm quality in Asian elephants (*Elephas maximus*). The present study aimed to investigate whether correlations among serum IGF-1 concentration, serum testosterone level and semen variables exist in elephants. A total of 17 ejaculates (1–3 ejaculates/bull) were collected by performing transrectal massage. Before each ejaculate, blood samples were obtained (n = 17). Subsequently, semen characteristics of each ejaculate were evaluated. Assessments of precision (calculations of the intra- and inter-assay coefficient of variation, CV) and accuracy (tests of linearity and spike-recovery) were performed for ELISA validation. An increase of serum IGF-1 concentration was found to correlate with the percentages of spermatozoa with intact acrosome ($r = 0.53$, $P < 0.05$) and normal head morphology ($r = 0.48$, $P < 0.05$). The serum IGF-1 concentration was positively correlated with serum testosterone level ($r = 0.73$, $P = 0.004$). The results of validation demonstrated the CV was 1.6–6.4% for intra-assay variability and 4.7–6.9% for inter-assay variability. Linearity under serial dilutions of a known serum concentration was confirmed ($R^2 = 0.99$) with an acceptable recovery rate of each dilution (mean 114.7 ± 19%, ranging from 100 to 143%). Additionally, the mean percentage of recovery of spiked IGF-1 was 107.2 ± 21% (ranging from 80.8 to 136.9%). In summary, this commercial ELISA kit can be used to determine serum IGF-1 concentration in Asian elephants. Moreover, our findings suggest the increased IGF-1 may be relate to good sperm quality and that the relationship between serum IGF-1 and testosterone concentration indicates a crucial role in the fertility of male elephants. Further works are interesting to investigate the exact mechanism by which IGF-1 affects semen quality in elephants.

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Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µL	Microliter (s)
µM	Micrometer (s)
AI	Artificial insemination
AO	Acridine orange
ARTs	Assisted reproductive technologies
BCS	Body condition score
CASA	Computer-assisted sperm analysis
cm	Centimeter (s)
Cl^-	Chloride
CPK	Creatine phosphokinase
CV	Coefficients of variation
dB	Decibel
DNA	Desoxyribonucleic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assays
FITC-PNA	Fluorescein-labeled peanut agglutinin
FSH	Follicle-stimulating hormone
g	Gravity
GH	Growth hormone
GHRs	Growth hormone receptors
GLU	Glucose
GnRH	Gonadotrophin-releasing hormone
HOST	Hypo-osmotic swelling test
HRP	Horseradish peroxidase
HPT	Hypothalamic-pituitary-testicular
IGF-1	Insulin-like growth factor-1
IGFBPs	IGF-1 binding proteins
IGFRs	IGF receptors

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IUCN	International Union for Conservation of Nature
kg	Kilogram (s)
LH	Luteinizing hormone
m	Meter (s)
min	Minute (s)
mL	Milliliter (s)
M	Molar (s)
MDA	Malondialdehyde
Na^+	Sodium
NaCl	Sodium chloride
Na_2HPO_4	Dibasic Sodium Phosphate
NEI	National Elephant Institute
ng	Nanogram
PBS	Phosphate buffered saline
PC	Protein carbonyls
pH	Negative logarithm of hydrogen ion concentration
PI	Propidium iodide
R^2	Coefficient of determination
RIA	Radioimmunoassay
s	Second (s)
SD	Standard deviation
TGF	Transforming growth factor
v/v	Volume per volume
y	year (s)

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CHAPTER 1

Introduction

Captive Asian elephants (*Elephas maximus*) are important not only for the tourism economy but also for improving the genetic diversity of the *ex-situ* population. However, despite the importance, the number of captive elephants dramatically decreased by 95% in the last century in Thailand (Kontogeorgopoulos, 2009). Moreover, a growing concern today is that the captive populations are not self-sustainable in ranging countries other than European groups due to a low birth rate, high mortality and imbalance of sex ratio (Thitaram, 2011; Toin et al., 2020). Furthermore, it has been challenged to obtain consistently good semen quality because high variations in ejaculate quality have been exhibited even in the same bull (Brown et al., 2004b; Thongtip et al., 2008; Kiso et al., 2011). While artificial insemination (AI) with fresh, chilled and frozen-thawed semen is suggested to reduce limitations of animal transportation over long distances and risks of natural mating (Brown et al., 2004b; Thongtip et al., 2009; Hildebrandt et al., 2012), improving poor semen quality is necessary to obtain successful conceptions in the elephants.

Insulin-like growth factor-1 (IGF-1) is located in the testis in which it is produced by Sertoli cells and Leydig cells, and it can modulate reproductive performance by stimulating steroidogenesis, cell proliferation and differentiation (Vannelli et al., 1988a; Cailleau et al., 1990; Lejeune et al., 1996; Roser, 2001; Griffeth et al., 2014). IGF-1 receptors have been identified in the spermatogonia (Le Gac et al., 1996), spermatocytes (Yoon et al., 2011), early spermatids (Vannelli et al., 1988b), spermatozoa (Henricks et al., 1998; Naz and Padman, 1999) and basal cells of the epididymis (Leheup and Grigong, 1993; Yoon et al., 2015) in several species. The above information suggests that the IGF-1 is involved in steroidogenesis and signals regulating spermatogenesis of the paracrine-autocrine system. Furthermore, serum

IGF-1 is simultaneously increased with testicular volume in men (Juul et al., 1994). The *Igf*1 gene encodes IGF-1, mice with an *Igf*1 null mutation are observed smaller sizes of testis and epididymis, lower levels of testosterone and sperm concentrations (Baker et al., 1996). A significantly lower level of serum IGF-1 is associated with semen abnormalities and prostate cancer (Baffa et al., 2000; Lee et al., 2016). Recently, the serum IGF-1 concentration in association with sperm concentration and motility has been reported in the buffalo bull (Kumar et al., 2019). Greater IGF-1 concentration in peripheral blood has been identified in the normal-semen group (normal sperm head morphology and motility) in Japanese black beef bulls during puberty age (Weerakoon et al., 2018).

Measurements of IGF-1 concentration are useful to understand possible factors relating to sperm quality and then to improve fertility rate. Nevertheless, so far, there is no information available regarding the role of IGF-1 in Asian elephant fertility. Therefore, the objectives of this study were to: (i) determine the relationships among serum IGF-1 concentration, testosterone level and semen variables, (ii) assess the validity of a commercially human IGF-1 enzyme-linked immunosorbent assay (ELISA) kit in detecting concentrations of serum IGF-1 in the Asian elephant bulls.

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CHAPTER 2

Literature Review

2.1 Status of population

Asian elephant (*Elephas maximus*) has been listed as an endangered species in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. In Thailand, the number of captive elephants dramatically decreased from 100,000 in 1900 to only 4,450 in 2009 (Kontogeorgopoulos, 2009). Recently, captive and wild elephants were estimated at less than 4,000, respectively, in Thailand. Maintaining the population of Asian elephants in a self-sustainable status has been focused on for decades. However, the reproduction rate is still low, possibly because of a low birth rate, high mortality, and skewed sex ratio (numbers of males < females) (Bansiddhi et al., 2018; Toin et al., 2020), although the use of elephants has been changed from logging to tourism in Thailand (Thitaram, 2011).

2.2 Reproductive anatomy

Testis morphology plays a crucial role in male reproductive status. The unique reproductive system of male elephants is the intraabdominal testis. The testis size of elephants varies with age, which has been identified in a previous investigation in which the diameter of immature testis ranges from 2 cm (newborn) to 9 cm (prepuberty) and mature testis is more than 10 cm (Hildebrandt et al., 2000a). The same authors found the testis size of older or dominant elephants is around 10 to 16 cm with consistent good semen quality. By comparison, inconsistent and inviable semen quality was obtained when elephants with testis diameters less than 10 cm (Hildebrandt et al., 2000a).

The maximum diameter of ampullae of Asian and African elephant bulls is estimated by ultrasound at 5 cm with a length of 6–8 cm (Hildebrandt et al., 2000b).

In Thailand, elephants' left and right ampullae range from 1.59–5.50 cm and 2.59–4.48 cm, respectively (Imrat et al., 2014). The ampullary diameters are positively correlated with the improvement of the level of motile, normal sperm morphology, and intact membrane spermatozoa after frequent stimulations; no correlation is found between age and ampullae size (Imrat et al., 2014).

Seminal vesicles are usually the largest accessory glands of a breeding bull. It can contain up to 400 mL of fluid in each gland in elephants. In a healthy elephant bull, the seminal vesicles combined 25–150 mL of fluid per ejaculation, up to five times per day, during natural breeding (Hildebrandt et al., 2000b). There is no evidence that prostate glands could be used as an infertility indicator. However, the only difference in sex assessor glands between African and Asian elephants is that Africans have the larger prostate gland. One previous study revealed that one or more accessory sex glands (ampullae, seminal vesicles, prostate gland, etc.) during the collection process might be influencing semen quality (Kiso et al., 2013).

A better understanding of the reproductive system is helpful to mitigate poor breeding problems in elephants. In addition, these criteria are useful to choose an optimal donor for breeding.

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2.3 Reproductive behavior

Mating behavior

Puberty of captive elephants is defined as 10 to 15 years of age and bulls during this period are capable of sperm production; however, bulls cannot successfully mate until 18 to 20 years old due to insufficient large body size to mount cows (Schulte, 2006).

Males advertise their reproductive status, physical states and locations to attract females and other males by vocalizing and giving off chemical signals.

Elephants can make low-frequency calls ranging 15–35 Hz at 5 m from the source (Poole et al., 1988). The low frequency of calls is used as a medium to coordinate their reproductive activities between individuals and group communications, such as cow-calf, males-males, females-males vocalizations because these calls can extend distances of several kilometers (Poole et al., 1988; Langbauer et al., 1991). When males perceive vocalizations from females, they will remain silent to approach males to not alert rivals and move toward females as quickly as possible (Poole, 1987). As a result, musth elephant bulls tend to be more dominant than non-musth bulls and they spend more time with females searching for mating opportunities and frequently vocalizing to attract females in estrus (Poole, 1987).

On the contrary, younger and smaller elephant bulls are likely to compromise the frequency of vocalizations. The size of males determines the success in male-male competition for female mating. Older musth elephants are more successful than youngsters at guarding receptive females because older bulls are larger and more aggressive (Poole, 1989). Estrous females are exercising choice to mate with old, vigorous and healthy males. Male bulls do not need to mate throughout the musth period, but females show preferences for high-ranking musth bulls to mate.

In addition to vocalization, wild Asian elephants use complex olfactory chemical signals to maximize reproduction success (Payne et al., 1986). During the preovulatory period, female Asian elephants release a higher level of the estrous pheromone (urinary pheromone (Z)-7-dodecenyl acetate), which peaks before ovulation. Musth wild bulls with behavioral responses such as temporal gland swelling/secretion can significantly detect periovulatory cows and precisely and assess their ovulatory status. These actions provide synchrony between sexes for successful reproduction (Rasmussen et al., 2005).

Musth

Elephant handlers or keepers are injured even died due to the disobedience and violence of musth elephants. More importantly, the human-elephant conflict is provoked. Considering that, the specific musth management for the captive and wild elephants are urgently necessary for ensuring the safety of humans, other animals and violence in the herd. In this chapter, the musth of elephants will be discussed to provide more information on this special reproductive status and emphasize managing elephant musth.

In elephants, the musth period is based on serum testosterone concentration in the elephants (Brown et al., 2007). In reindeers, the plasma insulin-like growth factor-1 reaches a peak at the rutting period (Bubenik et al., 1998). Captive bulls exhibit musth much earlier than those in the wild (Poole, 1987; Ganswindt, 2005; Ganswindt et al., 2010). Both Asian and African bulls exhibit a similar pattern of musth throughout a year in terms of behavioral (aggression; disobedience; decreases of appetite, etc.) and/or physical signs such as temporal gland secretion/swelling, urine discharge, penis discoloration and particular odor, etc. (Brown et al., 2007; Ganswindt et al., 2010). The musth duration is highly variable among one day to several months between or within an individual (Asian elephant: one to ten months (Brown et al., 2007), Africa elephants: one day to 127 days (Poole, 1987)).

However, the triggers of musth in the elephants are still not clear. Regardless of that, bulls frequently exhibit musth during high rainfall seasons and when interacting with estrus cows. Furthermore, many studies revealed a significant increase in androgen (Ganswindt et al., 2010; Chave et al., 2019). A distinguishing feature of musth elephants is a markable increase of testosterone level compared to non-musth elephants (Brown et al., 2007; Somgird et al., 2016; Chave et al., 2019). Moreover, luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid

hormone (total and free T4) production and cortisol secretion increase during the musth status (Ganswindt, 2005; Brown et al., 2007; Chave et al., 2019). Therefore, these are likely to be considered as a trigger of musth, but further investigations are needed.

The elephant facility needs to take housing, diet and health care into considerations for safe and proper musth management. Every musth cycle from beginning to the end needs managers to continuously and closely monitor individual bull. Non-invasive hormonal monitoring is necessary to predict and avoid unpredictable aggression and violence. Recently, vasectomy has been performed; but it does not impact musth behaviors (Zitzer and Boult, 2018). In addition to surgery, the leuprolide acetate (a gonadotrophin-releasing hormone (GnRH) agonist) has been used to control musth; however, the permanent and temporary damage to the reproductive organs still not evident. Also, vaccination against GnRH has been used and validated that aggression is improved by this treatment (De Nys, 2010). Although keeping elephants isolated in captivity until the end of musth is a very conventional method, it is, to some extent, a safe and less costly approach.

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2.4 Semen characteristics

Sperm physiology

The dimensions of Asian elephant (*Elephas maximus*) spermatozoa were detected using computer-assisted sperm analysis (CASA) with an estimated length of 7.5 μm major, 3.8 μm minor, and 57.8 μm elongation. The size for the sperm head of the Asiatic elephant (*Elephas maximus*) are estimated at 7.8 μm in length and 4.7 μm in width, and 47.3 μm for the sperm tail (Heath et al., 1983). Compared to most mammals, sperm cells and bodies of elephants are smaller (Cummins and Woodall, 1985).

Seminal characteristics with 226 ejaculates of Asian elephants have been demonstrated, including volume, sperm concentration, progressive motility, sperm viability, pH (Thongtip et al., 2008), which provides more general information for the physiology of elephant sperm.

The most abundant membrane fatty acid is docosahexaenoic acid in the spermatozoa of elephants (Swain and Miller Jr, 2000). However, there is a higher percentage of membrane docosahexaenoic acid in the African spermatozoa than Asian elephants, which may cause the differences in reacting to the freezing procedure. Images of Asian elephant spermatozoa evaluation using different methods shown in Fig. 1–Fig. 4. Images were taken by Yuqing.

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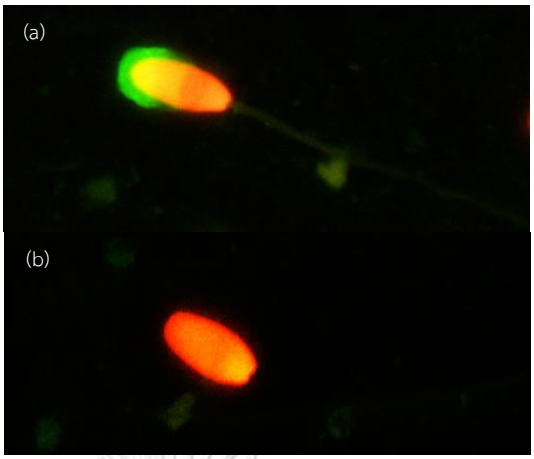

Figure 1 Asian elephant sperm acrosome was assessed using FITC-PNA/PI staining under a fluorescent microscope at a magnification of 1000 ×. a). Acrosome-intact: spermatozoa with bright green fluorescence of the acrosomal cap; b). Acrosome-missed: spermatozoa with a fluorescent band at the equatorial segment or no fluorescence.

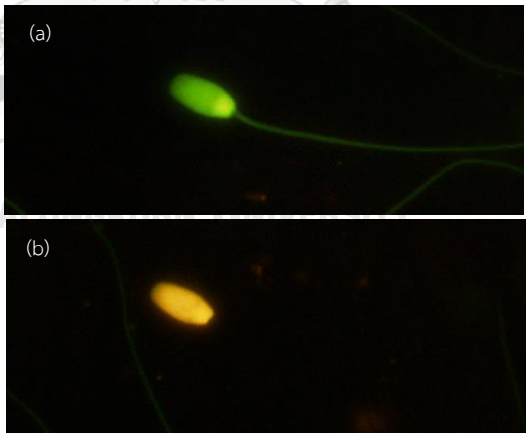

Figure 2 Asian elephant sperm acrosome was assessed using Coomassie blue staining under a light microscope at a magnification of 1000 ×. a) Acrosome-intact: a clear demarcation of acrosome in the apical region of sperm head; b) Acrosome-reacted: negligible (faint or no dark) staining in the acrosomal region.

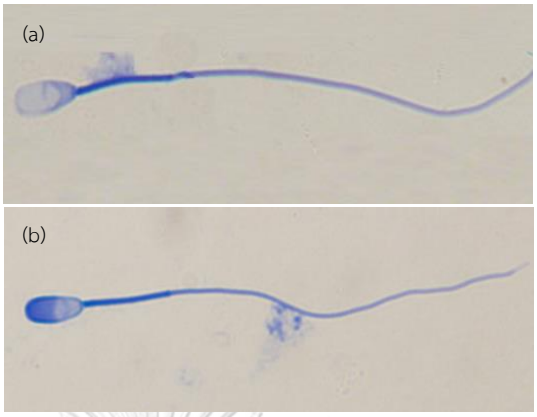

Figure 3 Asian elephant sperm DNA was assessed using Acridine orange (AO) staining under a fluorescent microscope at a magnification of 1000 ×. a). Normal: spermatozoa head with green fluorescence (double-stranded); b). Abnormal: spermatozoa stained with red, yellow, orange, or mixed fluorescence

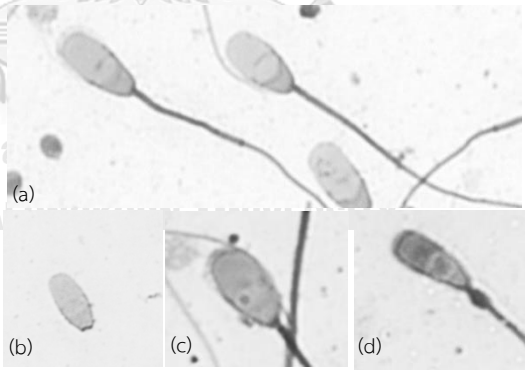

Figure 4 Asian elephant sperm head morphology was assessed using William staining under a light microscope at a magnification of 1000 ×. a). Normal; b). Loose; c). Round; d). Narrow.

Seminal plasma

Seminal plasma is a critical element of semen quality and an essential and potential source of male fertility. Malondialdehyde (MDA) and protein carbonyls (PC) are important biomarkers for detecting lipid and protein oxidation (Saraniya et al., 2008; Shiva et al., 2011). Seminal MDA and PC of Asian elephants have been reported as being higher in the good (motility > 60%) than poor semen (motility < 20%) quality group (Satitmanwiwat et al., 2017), suggesting oxidative processes may affect elephant sperm motility. A previous study has reported that 85% of Lactotransferrin presents good sperm motility, suggesting Lactotransferrin may be a key point for elephant semen preservation (Kiso et al., 2013). Also, the same authors found that seminal CPK, Na^+, Cl^-, and GLU are also highly presented in sperm with good motility. Furthermore, seminal plasma derived from stallions can maintain the sperm motility of Asian elephants during the cooling process (Pinyopummin et al., 2017). Nevertheless, the studies of seminal plasma in elephants are limited.

2.5 Poor semen quality

The semen quality of Asian elephants is categorized into good and poor semen quality based on sperm motility in the previous investigations (Kiso et al., 2013; Satitmanwiwat et al., 2017). Reproductive failure in elephants is caused by females and/or males (Hildebrandt et al., 2000). In the aspect of male elephants, inconsistent and unpredictable sperm quality has been confirmed. Specifically, significant variations have been observed within and/or between ejaculate quality even in the same individual bull, although the volume of ejaculate is far more enough (Brown et al., 2004; Thongtip et al., 2008; Kiso et al., 2011), which possibly causes low pregnancy rate of elephants. Factors contributing to poor semen quality in the elephants are not fully understood.

Semen can be collected from elephants by using various methods, including rectal massage (Schmitt and Hildebrandt, 1998), electroejaculation (Howard et al., 1984), and artificial vagina (Hildebrandt et al., 2000b). Transrectal massage without sedation is a safe and practical method. Therefore, it is commonly used for routine semen collection from elephant bulls nowadays; however, sperm quality collected by this method is generally poor, especially with high levels of DNA damage (O'Brien et al., 2013).

In Thailand, the lowest semen quality is obtained during summer and the highest is obtained in the rainy season even though the elephant is a non-seasonal breeder (Thongtip et al., 2008). Age-associated semen quality has been reported that the greatest semen quality is found in 23–43-year-old group. In contrast, the semen concentration is the lowest during the age from 10 to 19 years old in Asian elephant bulls (Thongtip et al., 2008). Moreover, the accumulation of senescent semen seems another main factor causing poor semen quality in the elephants. It was previously reported that the levels of the motile and intact membrane, normal morphology spermatozoa are increased after frequent stimulations (five times on alternate days). In the same study, semen pH is increased from neutral to alkaline with higher ejaculate motility after repeated semen collections (Imrat et al., 2014).

Intraabdominal testis of elephants means no pampiniform plexus can cool down the body temperature. As a result, the average body temperature of elephants is 34–36 °C. In particular, Asian elephants live in tropical weather in Thailand. This uniqueness has been suspected as the one factor contributing inconsistent semen quality of elephants, but the underlying reasons are not validated.

2.6 Assisted reproductive technologies (ARTs)

Artificial insemination (AI)

Due to restrictions on the transportation of endangered species across countries, captive breeding management is currently crucial to conserve genetic diversity. In addition, assisted reproductive technologies (ARTs) help minimize the violence during natural mating and risks of disease transmission between males and females (Hildebrandt et al., 2006).

Artificial insemination (AI) has been validated and used as an alternative tool for enriching the captive population genetic pool in this endangered species. However, only one study reported AI with frozen-thawed semen successfully produced the birth of Asian calve (Thongtip et al., 2009). Factors contributing to success AI or success pregnancy were complex, which depends on screening and providing good quality semen during the estrus cycle. Specifically, this including the use of hormone monitoring (Enzyme immunoassay [EIA] and Radioimmunoassay [RIA]) of progesterone or luteinizing hormone (LH) in the females to determine the accurate timing (conducting several inseminations at 18-22 days later to coincidence with LH2) for AI and the delivery of semen into the anterior vagina (Brown et al., 2004b; Thongtip et al., 2009; Hildebrandt et al., 2012).

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Semen (cryo)preservation

Successful semen preservation can maximize the utility of AI by using appropriate extenders to reduce the metabolic activity of spermatozoa of elephants during the cooling (chilling or freezing) process (Kiso et al., 2011). Fragile semen of Asian elephants during cryopreservation is not difficult to find, and it has faced many obstacles and difficulties compared to African elephants. Many attempts have been reported to discover the underlying factors causing disappointing semen in Asian

elephants. For example, the dilution ratio affects elephant sperm motility (Pinyopummin et al., 2018). Also, pretreatments make semen preservation possible because cooling, dilution, and post-thaw processes adversely impact on sperm DNA integrity when incubating elephant semen at 37 °C (Imrat et al., 2012). Thus, it implies that it is vitally necessary to perform carefully in every (cryo)preservation process in which DNA damage should be avoided as much as possible. Even though sperms are sensitive to the components of different semen extenders in Asian elephants, many extenders, cryoprotectant and cryopreservation protocol have been recommended (Thongtip et al., 2004; Kiso et al., 2011; Kiso et al., 2012; Buranaamnuay et al., 2013; Imrat et al., 2013). Moreover, a field-friendly technique for cryopreserving semen in Asian elephants has been discovered in a recent report in which faster rates of freezing and thawing (at temperatures of 50 °C or 75 °C) together with the use of a dry shipper are recommended to increase the efficacy and practicability of semen cryopreservation (Arnold et al., 2017). Nevertheless, making effective semen (cryo)preservation protocols in this field needs more investigation.

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2.7 Insulin-like growth factor-1 (IGF-1)

The IGF-1 is a 70 amino acid peptide. It is secreted primarily by the liver and found in all body tissues and fluids (Griffeth et al., 2014). The IGF system, which consists of IGF-1 and IGF-2 ligands, is part of a major growth-promoting signaling system involved in embryonic and postnatal development. Moreover, IGF-1 is vital for measuring fetal body growth and is produced at low levels during the embryonic period. Thus, IGF-1 is considered more important for adult growth/development than other insulin family members (Dupont and Holzenberger, 2003). Thus, the baseline of serum IGF-1 concentration has the potential to be a diagnostic tool to measure postnatal developments and nutritional status in captive endangered species or even those in the wild (Govoni et al., 2011).

IGFs are the essential growth factors in regulating the number of Sertoli cells and size of testis (Pitetti, 2013). Testis size and sperm production are directly correlated to the total number of adult Sertoli cells (Orth et al., 1988). IGF-1 has been identified in the testis (Vannelli et al., 1988a; Yagci and Zik, 2006; Yoon et al., 2011), where it is produced by Leydig cells and Sertoli cells (Lin et al., 1990; Roser, 2001). IGF-1 receptors (IGF-1Rs) have been identified in the spermatogonia, spermatocytes, early spermatids, spermatozoa, Sertoli cells, and Leydig cells (Vannelli et al., 1988; Le Gac et al., 1996; Henricks et al., 1998; Naz and Padman, 1999; Yoon et al., 2011) and cells of the epididymis (Leheup and Grigong, 1993; Yoon et al., 2015). In the seminal plasma, IGF-1 has been investigated in boar (Hirai et al., 2001; Zangeronimo et al., 2013), bovine (Henricks et al., 1998), equine (Yoon et al., 2011), human (Naz and Padman, 1999; Lee et al., 2016), rabbit (Minelli et al., 2001), and fish (Le Gac et al., 1996). All the above evidence is supporting that IGF-1 is essential in signal-regulating spermatogenesis in mammals.

Spermatogenesis is a complex physiological process in which spermatogenic cells are influenced by testosterone and pituitary gonadotrophins through the endocrine-paracrine-autocrine systems (De Kretser et al., 1998). Several external and internal factors, such as seasons, puberty and protein factors, have been proposed as being associated with the process of spermatogenesis, resulting in cellular activities (Gnessi et al., 1997; Roser, 2001). Male reproduction depends upon local paracrine and autocrine factors such as IGFs, transferrin, transforming growth factor (TGF), steroid hormones (testosterone), and classic endocrine gonadotropins (LH and FSH). All factors coordinate together to control spermatogenesis under the hypothalamic-pituitary-testicular (HPT) axis (Gnessi et al., 1997).

2.8 IGF-1 and male infertility

Serum IGF-1 has simultaneously increased with testicular volume in men (Juul et al., 1994). IGF-1 is encoded by the *Igf*1 gene, mice with an *Igf*1 null mutation are around 30% smaller, have reduced weights of the testis, epididymis, and testosterone and sperm concentrations are less than a quarter of normal control groups (Baker et al., 1996). Moreover, the first cycle conception rate of stallions females is negatively correlated to seminal IGF-1 concentration of males, suggesting that IGF-1 is an independent biomarker of infertility (Novak et al., 2010).

The IGF-1 level is related to male infertility; however, it is varied among species. In bovines, the mean (± SD) serum IGF-1 concentration was estimated as 116.29 ± 40.83 ng/mL detected by RIA (Henricks et al., 1998). Also, the same author found sperm motility was improved by the interaction between IGF-1 and its receptor at the sperm acrosomal region and the supplementation of IGF-1 (250 ng/mL), which maintains good semen motility compared to the control group (IGF-1:100 ng/mL) (Henricks et al., 1998). Men with normal semen parameters had different levels of serum and seminal IGF-1 concentrations (175.4 ± 42.7 and 15.4 ± 8.3 ng/mL, respectively); accordingly, men with abnormal sperm parameters had a lower level of IGF-1 (137.6 ± 27.1 and 17.0 ± 5.2 ng/mL, respectively) (Lee et al., 2016). In buffalo bulls, there is a positive correlation between IGF-1 concentration in serum (mean, 1555.22 ng/mL; range, 927 to 2247 ng/mL) and sperm motility and concentration, respectively (Kumar et al., 2019).

In boars, the addition of IGF-1 into seminal plasma (mean, 1.5 ± 0.20 ng/mL; range, 1.39 to 2.44 ng/mL) can maintain sperm longevity (Zangeronimo et al., 2013). During the cooling storage of ram semen, the supplementation of IGF-1 significantly improves spermatozoa motility and membrane integrity (Makarevich et al., 2014). In the buffalos, seminal IGF-1 positively affects sperm motility, plasma membrane integrity, lipid peroxidation, and fructose uptake *in vitro* (Selvaraju et al., 2009).

To date, only one study published the concentration of serum IGF-1 detected by RIA in seven threatened hoofstock species, including male Asian elephants (307 ± 52 ng/mL) (Govoni et al., 2011).

2.9 Factors that affect IGF-1 concentrations

IGF-1 is stimulated by testosterone (Itoh et al., 1994), FSH (Cailleau et al., 1990; Itoh et al., 1994), LH (Lejeune et al., 1996). The synthesis and release of testicular IGF-1 are mediated by growth hormone (GH) (Chandrashekar et al., 1999). The absence of growth hormone receptors (GHRs) and IGF-1 secretion is associated with reduced body weight and testicular function (Chandrashekar et al., 1999). GH and IGF-1 act at pituitary gonadotrophs and gonadal sites to modify GnRH actions, influencing gonadal functions (Choubey, 2020). In the Sertoli cells, FSH amplifies IGF-1 to mediate PI3K/AKT signaling; FSH also enhances PI3K/AKT signaling (Khan et al., 2002). FSH and IGF-1 pathways are intimately connected in animals (Cannarella et al., 2018). Thus, factors influencing the productions of gonadotrophins and GH may also affect the IGF-1 level.

Besides, IGF receptors (IGFRs) and IGF-1 binding proteins (IGFBPs) are significantly recognized in the IGF family (Jones and Clemmons, 1995). The majority of actions of IGF-1 such as cell cycle progression, cell proliferation and cell death are mediated by IGF-1R (Le Gac et al., 1996; Naz and Padman, 1999; Yoon et al., 2011; Yoon et al., 2015). In addition, IGFBP-2 (Macpherson et al., 2002), IGFBP-3 (Plymate et al., 1996), IGFBP-5 (Macpherson et al., 2002) and IGFBP-6 at least six high-affinity binding proteins coordinate together and regulate the biological activities of the IGFs. Furthermore, IGFBPs can inhibit or enhance IGFs actions. Thus, the bioavailability and effects of IGFs are also controlled by IGFBPs.

Nutritional status plays an influential role in IGF-1 levels. Levels of serum and seminal plasma IGF-1 are higher when rams are fed with high-energy diets than the low-energy dietary group (Selvaraju et al., 2012). Enrichment of n-3 PUFA in the buffalo bull diet significantly increases levels of IGF-1 and testosterone (Tran et al., 2016). On the other hand, overweight elephants are not difficult to find in the zoo or captivity. Up to 39% of the population of Asian elephants reaches high body condition scores (4.0–5.0) in Thailand (Norkaew et al., 2018) as well as 74% of the population in both African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants in North American zoos is overweight (Morfeld et al., 2016). Thus, IGF-1 has the potential to be a measurement tool for monitoring nutritional status and screening obesity of elephants.

IGF-1 concentration is related to age, particularly the age of puberty, and it has been shown to support the development and maintenance of spermatogenesis (Hess and Roser, 2001). During puberty, the strong contents of testicular IGF-1 and IGF-1R have been shown in Leydig cells and spermatogonia in the stallion testes (Yoon et al., 2011). The previous study has been demonstrated that high plasma and testicular IGF-1 levels are exhibited in the stallions younger than two years old and decrease until more than five years old, suggesting that IGF-1 may be under the control of or regulate testicular development (Hess and Roser, 2001). The same result has been shown in men, demonstrating that the growth factor is known as IGF-1, which depends on ages; it gradually increases until 13-15 years old, but after puberty, it ultimately decreases (Salardi et al., 1986).

2.10 Testosterone

Testosterone is secreted by Leydig cells, and it plays a key role in several male reproductive functions, including extragonadal actions for sexual (libido) and anabolic

(muscle strength, bone density) functions (Dutta et al., 2019). Additionally, it has been clear that testosterone is essential in the maintenance of qualitatively semen quality by playing imperative roles in spermatogenesis, sperm maturation and sperm release. And testosterone improves sperm motility, facilitates spermatogenesis locally in the Sertoli cells as a paracrine factor, and provides feedback for GnRH and LH secretion (Meeker et al., 2007).

Testosterone significantly increases both IGF-1 and IGFBPs, stimulating cell proliferation by a complex process (Ashton et al., 1995). As demonstrated in other species, circulating serum IGF-1 concentration is associated with testosterone (Ditchkoff et al., 2001; Brito et al., 2007).

In the male elephants, the overall mean testosterone concentration and its baseline of Asian elephant bulls have been measured at 33.40 ± 1.88 and 1.12 ± 0.39 ng/mL, respectively (Brown et al., 2007). Age can influence serum and seminal plasma testosterone in Asian elephant bulls. (Thongtip et al., 2008). During the musth period, testosterone exceeds 50 ng/mL (Brown et al., 2007). GnRH induces LH secretion and is followed by testosterone secretion during the musth period other than the pre-musth and post-musth periods (Somgird et al., 2016).

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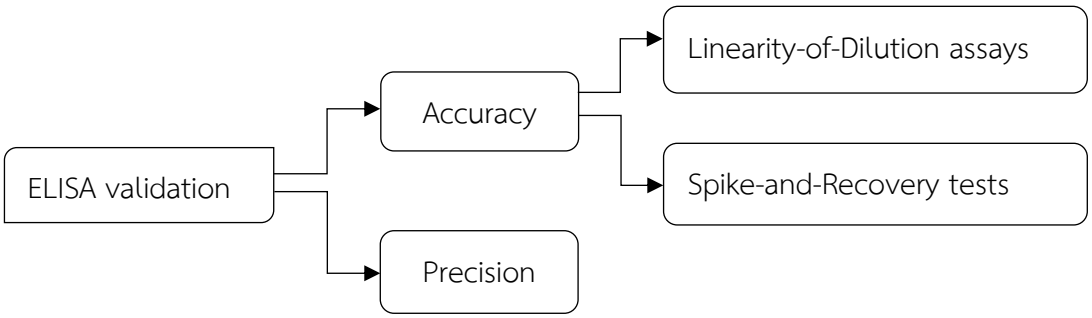
2.11 Enzyme-linked immunosorbent assay (ELISA) validation

ELISA validation is an evaluation process to determine its fitness for a particular use. The assessment of ELISA validation is designated to combine dilutional linearity assay and spike-and-recovery test simultaneously (Scientific, 2007). Linearity-of-dilution assays and spike-and-recovery tests are the most common and essential methods to validate and assess the ability of the ELISA in measuring the true or accurate amount of target analyte in the sample (Kragstrup et al., 2013; Axnér and Holst, 2014).



Linearity-of-dilution

A linearity-of-dilution assay is used to demonstrate an accurate linear detection of examined target analyte concentrations, representing accuracy and reliability in the ELISA (Wang et al., 2012; Stiller et al., 2016). During this process, a known concentration sample is diluted under serial dilutions such as 1:2, 1:4, 1:8 and other subsequent dilutions. Suppose the sample results do not show linear dilution indication matrix component, which interferes with detecting the accurate target analyte under the specific dilution in the ELISA. The percentage recovery of each dilution ranging from 80% to 120% is acceptable and it is calculated by dividing the observed values by the expected values and multiplying by 100.

% Recovery of dilution (1:2) = con (analyte at dilution 1:2) / con [neat])

% Recovery of dilution (1:4) = con (analyte at dilution 1:4) / con (analyte at dilution 1:2)

% Recovery of dilution (1:8) = con (analyte at dilution 1:8) / con (analyte at dilution 1:4)

etc. for all other subsequent dilutions.

Spike-and-recovery

A spike-and-recovery test refers to the accuracy and reliability of the ELISA. It is used to investigate whether a component in the sample interferes with the ELISA (Stiller et al., 2016). A spike-and-recovery test is performed to examine whether analyte detection is affected by a difference between the diluent used to prepare the standard curve and the experimental sample matrix (Scientific, 2007). A known amount of analyte is spiked with a test sample matrix and its response is measured by testing this sample against an identical spike in the standard sample diluent in the ELISA. The result of spike concentration is recovery.

% Recovery= [con (observed) – con (neat)] / con (expected)

Precision

A precision test is a measurement of the dispersion of results for a repeatedly tested sample. A small amount of dispersion indicates a precise assay. Precision is measured by calculating intra-assay coefficients of variation (CV) and inter-assay CV. Intra-assay CV is used to determine the within assay error (the error associated with running the same sample in one assay) and inter-assay CV is used to determine the between assay error (the error observed when the same sample is run in different assays) (Brown et al., 2004c). The CV is calculated:

Coefficient of variation = (SD/mean) * 100%

2.12 Hypothesis

There are relationships among levels of serum IGF-1, serum testosterone and semen variables of Asian elephants.

2.13 Objectives

(1). To determine the relationships among serum IGF-1 concentration, testosterone level and semen variables in the Asian elephant bulls.

(2). To assess the validity of a commercially human IGF-1 enzyme-linked immunosorbent assay (ELISA) kit in detecting concentrations of serum IGF-1 in this endangered species

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CHAPTER 3

Materials and Methods

3.1 Animals

The study was approved by the Animal Care and Use, Faculty of Veterinary Science, Chulalongkorn University, Bangkok (approval number 2031044).

Seven healthy captive-born Asian elephant bulls, aged from 12 to 46 years (averaged 28.9 ± 13.5 years) with a mean body weight of 3357 ± 512 kg, housed at the National Elephant Institute (NEI), Forest Industry Organization Lampang, Thailand (latitude 18° 21.60′E and longitude 99° 14.92′E) were included in the present study. All elephant bulls were maintained in mixed social groups with females working for tourists either riding (with a saddle) or showing no more than 4 hours per day. When not working, the elephants were fed with Napier grass (*Pennisetum purpureum*), pangola grass (*Digitaria eriantha*), tamarind (*Tamarindus indica*), banana and other natural foods. The availability of foods varied with seasons. After working, all bulls were chained separately with long 20–30-m chains in the forest for foraging (from 16:00 to 18:30). Routine health examination was performed by veterinarians three times a year.

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3.2 Blood sampling

Blood samples (3–6 mL; n = 17) were collected from elephant ear veins between 9:00 to 10:00 a.m. before each ejaculate of the semen collection procedure. Samples were centrifuged at 2,000 × *g* for 10 min. Serum samples were harvested and stored at -20 °C until analysis.

3.3 Semen collection and evaluation

Seventeen ejaculates (one to three ejaculates per bull) were obtained from seven bulls by performing rectal massage as previously described (Schmitt and Hildebrandt, 1998). Ejaculates with the visualized or olfactory scent of urine contamination were excluded. Each ejaculate was evaluated immediately for color, volume and pH. For the percentages of motility and progressive motility, an aliquot of 5-µL of each ejaculate dropped on a pre-warmed glass slide (37 °C) and covered with a pre-warmed coverslip was subjectively estimated under a phase-contrast microscope at a magnification of 200 × (Olympus, Shinjuku, Japan) by three experienced evaluators. A hemocytometer chamber (Boeco, Hamburg, Germany) was used to determine sperm concentration.

3.3.1 Sperm membrane integrity

Eosin-nigrosin staining was used to assess the percentage of viable sperm. A total of 200 spermatozoa per sample was evaluated. Spermatozoa with no staining were classified as intact membrane (or live spermatozoa) and spermatozoa stained pink were classified as non-intact membrane (or dead sperm) (Björndahl et al., 2003).

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3.3.2 Sperm morphology

Sperm head morphology was assessed using William's staining (Lagerlöf, 1934). Five hundred spermatozoa per sample were examined under a light microscope (Olympus, Japan) at a 1000 × magnification with oil immersion. Abnormal sperm head morphology included the narrow head, pear shape, giant/broad/round/small head, abnormal contour, abnormal acrosome, abaxial, undeveloped, loose. For sperm tail morphology, an aliquot of the mixture of 5 µL

semen with formal saline solution (1:10) was dropped on a microscopic slide. Then, two hundred spermatozoa per sample were assessed under a phase-contrast microscope at 400 × magnifications by counting the amount of proximal droplet, distal droplet, simple bent, coil, abnormal midpieces, loose and normal tail.

3.3.3 Functional membrane integrity

The functional integrity of sperm plasma membrane was determined using a short hypo-osmotic swelling test (sHOST) (Pérez-Llano et al., 2001; Buranaamnuay et al., 2013). Briefly, each ejaculate (20 µL) was incubated at 37 °C in 200 µL hypo-osmotic solution (75 mOsm/kg; 1:10, v/v) for one hour. After incubation, the semen was fixed in a hypoosmotic solution supplemented with 5% (v/v) formaldehyde (Merck, Darmstadt, German). A drop (5 µL) of the mixture was placed on a warm slide (37 °C) and covered with a coverslip. A total of 200 spermatozoa per sample were counted under a phase-contrast microscope (400 ×). Coiled-tail spermatozoa presented as intact functional membrane integrity (sHOST positive).

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3.3.4 Sperm DNA integrity

Sperm DNA integrity was examined on Acridine orange (AO) fluorescence by modifying previous reports (Tejada et al., 1984; Thuwanut et al., 2008). Briefly, a thin smear of 8-10 µL of diluted semen onto a slide and air-dried on the slide warmer (37 °C). The slides were fixed in Carnoy's solution (methanol: glacial acetic acid, 3:1 v/v) overnight at room temperature and air-dried again. The smeared slides were stained with 1% (100 mg/mL) AO for 10 min. The AO staining solution was prepared daily by adding 10 mL of 1% AO to 40 mL of 0.1 M citric acid (Merck, Darmstadt, Germany) and 2.5 mL of 0.3 M $Na_2HPO_4 \cdot 7H_2O$ (Merck, Darmstadt, Germany) and then stored at the room temperature at the dark. After staining, the slides were gently rinsed in a

stream of distilled water. Two hundred spermatozoa per slide were examined using a fluorescent microscope (BX51, Olympus, Japan) at a 1000 × magnification with oil immersion. Spermatozoa head with green fluorescence was considered as normal DNA integrity (double-stranded), whereas those spermatozoa stained with the red, yellow, orange, or mixed fluorescence were considered as abnormal (Liu and Baker, 1992).

3.3.5 Acrosome integrity

The intactness of sperm acrosome was evaluated using fluorescein isothiocyanate-labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA) combined with propidium iodide (PI) staining (Axnér et al., 2004). Briefly, 10 µL FITC-PNA (1 mg/mL) was diluted with 90 µL phosphate buffer saline (PBS) and mixed with 5 µL of 340 µM PI. A circle smear of 5 to 8 µL of diluted semen was performed on a clean slide and air dried. The sample was fixed in 95% ethanol (v/v) for 30 s and allowed to air dry. Subsequently, spread a 50 µL mixture of FITC-PNA/PI over the smeared slide. After incubation for 30 min in a dark humidified chamber at 4°C, slides were rinsed with cold distilled water and air-dried. Two hundred spermatozoa per slide were examined under a fluorescent microscope at a 1000 × magnification immersion oil. Only acrosome-intact sperm were stained with bright green fluorescence (Cheng et al., 1996; Axnér et al., 2004).

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3.4 Validation and evaluation of serum IGF-1

Serum IGF-1 concentrations were determined by a commercially sandwich enzyme-linked immunosorbent assay (Human IGF-1 ELISA E20, Mediagnost, Germany). Before running the assay, serum samples were diluted with sample buffer at a 1:21 dilution ratio according to the manufacturer's protocol. Briefly, 80 µL of goat

biotinylated anti-human IGF-1 antibody was added to each well first. Subsequently, 20 µL of blank, controls, standards (recombinant human IGF-1 (hIGF-1)) and diluted samples were added in the wells of the microtiter. The plate was incubated for one hour at room temperature and washed firstly with washing buffer 5 times. After washing, horseradish peroxidase (HRP)-labelled Streptavidin was added to each well. After 30-min incubation and washing again, HRP-substrate was added and incubated for 15 min. The reaction was terminated by adding sulphuric acid solution. The absorbance was measured at 450 nm immediately using an ELISA plate reader (Tecan Sunrise™, Mannedorf, Switzerland).

The ELISA validation included assessments for accuracy (linearity-of-dilution assay and spike-and-recovery test) and precision (intra-, inter-assay coefficient of variation [CV], and total imprecision assay CV). Intra-assay and inter-assay CV were calculated from low, medium and high concentrations of samples. The inter-assay CV was calculated by analyzing these three samples duplicated in 4 separate plates on 4 different days. The intra-assay CV was evaluated from CV of the same samples in duplicates in the same plate. The total imprecision assay CV (CV_{total}) was calculated: $CV_{total} = \sqrt{CV(inter)^2 + CV(intra)^2}$. Desirable and acceptable CV_{total} were set at 8.5% and 12.75%, respectively (Stiller et al., 2016).

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One serum was evaluated first for demonstrating the concentration of IGF-1 (254.6 ng/mL). The linearity under dilution was evaluated by diluting (1/2 to 1/8) this serum sample with the PBS. Expected values were calculated by dividing the concentration from the undiluted sample by the dilution factor used (Axnér and Holst, 2014). The percentage of recovery for each dilution is assessed by comparing observed vs. expected values and multiplying by 100.

For the spike-recovery test, five low-concentration serum IGF-1 samples were pooled. The pooled sample was then spiked with 100 µL of 2, 5, 15, and 30 ng/mL

of standard recombinant hIGF-1 in duplicates, respectively. Recovery of spiked IGF-1 was calculated follow a previous study: [spiked sample (ng/mL) / (neat (ng/mL) + spike solution (ng/mL)] * 100 with an acceptable recovery ranged from 80% to 120% (Jaedicke et al., 2012; Stiller et al., 2016).

3.5 Evaluation of serum testosterone

Concentrations of serum testosterone in the Asian elephants were analyzed using an enzyme immunoassay (EIA) following the previous study (Brown et al., 2004a). Inter- and intra-assay CV were less than 10%.

3.6 Statistical analyses

Statistical analyses were performed using SPSS (IBM SPSS statistics version 22.0.0.0, SPSS Inc., Chicago, IL, USA). The normality of distribution was measured by the Kolmogorov Smirnov test and confirmed by Qualitative-Quantitative plots (Q-Q plots). The levels of serum testosterone and IGF-1 with semen analysis results were presented using descriptive statistics. Pearson's correlation coefficient (*r*) was performed to find correlations between IGF-1 level and all variables. All data were presented as mean ± SD, and the significance was set at $p < 0.05$ with a confidence interval of 95%.

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CHAPTER 4

Results

The background information including the mean serum IGF-1 concentration of seven Asian elephant bulls is summarized in Table 1. Overall mean (± SD) concentration of serum IGF-1 was estimated at 326.3 ± 114.6 ng/mL (ranging from 167.4 to 542.7 ng/mL). Large variations of IGF-1 concentration were exhibited among seven bulls. The youngest bull (E1) had high IGF-1 concentration while the oldest bull (E4) had the lowest IGF-1 level (Fig. 5).

Table 1 Summary of age, body condition score (BCS), body weight, ejaculate number, mean serum IGF-1 concentration and fertility history of Asian elephant bulls (n = 7)

ID	Age (y)	BCS	Body weight (kg)	Ejaculate number	Serum IGF-1 (ng/mL)	Fertility history
E1	12	3	2690	2	437.6	No
E2	20	3.5	3490	4	300.4	No
E3	27	3.5	4055	2	271.4	No
E4	45	3	3460	4	215.0	No
E5	27	3	3000	2	283.9	No
E6	46	3	3210	2	541.6	Yes
E7	40	3	4070	1	323.7	Unknow

Body condition score (BCS): 5-point scale (1–5, thinnest–fattest); 3–3.5 was defined as normal BCS.

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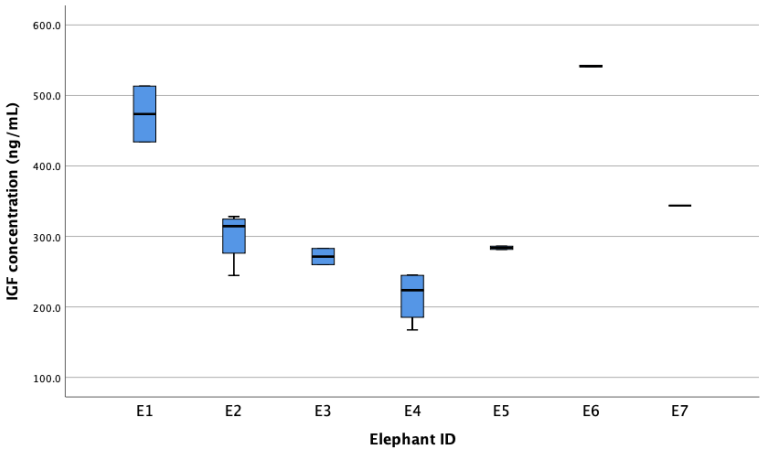

Figure 5 The distributions of serum IGF-1 concentration among seven Asian elephant bulls.

The mean values for all variables and correlations between IGF-1 concentration and all variables in this study are shown in Table 2. There was a positive correlation between serum IGF-1 and testosterone concentration (r = 0.73, P = 0.004, Table 2). Also, positive correlations between acrosome integrity (r = 0.53, P = 0.028) and the percentage of normal head morphology of spermatozoa (r = 0.48, P = 0.049), and IGF-1 concentration were identified.

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Table 2 Mean values and correlation between variables and serum IGF-1 concentration in seven elephant bulls (n = 17 ejaculates)

Variables	Mean	Serum IGF-1 correlation coefficient (*r*)	P value
Ejaculate volume (mL)	32.1 ± 13.3	0.12	0.66
Semen pH	7.3 ± 0.7	0.27	0.29
Sperm concentration ($\times 10^6$/mL)	1209.2 ± 403.4	0.07	0.80
Motility (%)	32.6 ± 22.8	0.09	0.74
Progressive motility (1–5)	3.2 ± 1.4	-0.15	0.58
Sperm membrane integrity (%)	58.6 ± 21.0	0.07	0.80
Normal sperm tail morphology (%)	71.9 ± 15.8	-0.14	0.60
Normal sperm head morphology (%)	64.0 ± 21.2	0.48	0.049*
Intact functional membrane integrity (%)	26.5 ± 16.8	0.09	0.74
Normal sperm DNA integrity (%)	70.0 ± 23.5	0.13	0.61
Intact acrosome (%)	44.5 ± 18.3	0.53	0.028*
Serum Testosterone (pg/mL)	1190.1 ± 1081.8	0.73	0.004**
Serum IGF-1 (ng/mL)	326 ± 114.6	-	-

* indicates P < 0.05

** indicates P < 0.01

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The linearity under the serial dilutions was conformed (R^2 = 0.99; Fig. 6) in the ELISA and the recovery rate ranged from 100 to 143% with a mean of 114.7 ± 19%, which were acceptable.

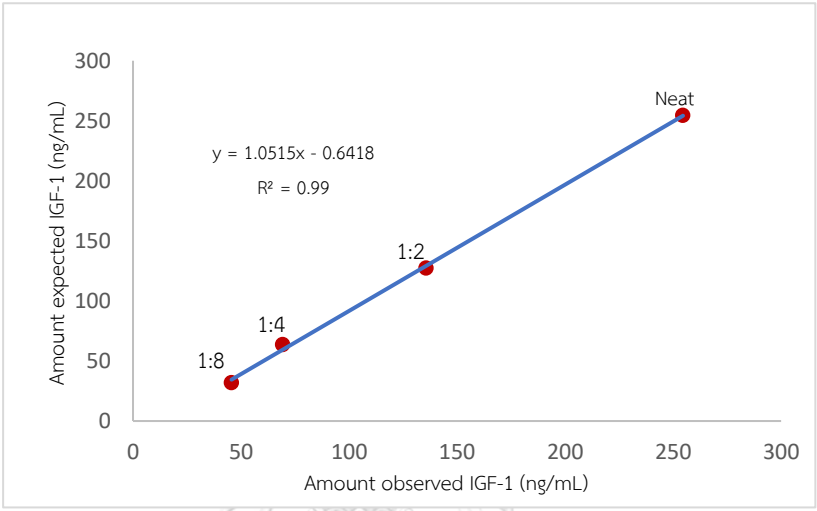

Figure 6 Investigation of linearity through a serial dilution of one serum sample for demonstrating an accurate linear detection of examined insulin-like growth factor-1 (IGF-1) concentrations.

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Inter-assay, intra-assay and total imprecision CV for low, medium and high concentration samples were 1.6–6.4% and 4.7–6.9%, 7.1–8.0%, respectively (Table 3). In spike-recovery assays, the overall mean recovery of spiked IGF-1 was acceptable (mean 107.2 ± 21%, range 80.8–136.9%).

Table 3 Inter-assay, intra-assay and total imprecision coefficients of variation of serum IGF-1 samples with low, medium and high concentration

Sample concentration	Intra-assay CV			Inter-assay CV			
	Mean (ng/mL)	SD (ng/mL)	CV (%)	Mean (ng/mL)	SD (ng/mL)	CV (%)	CV_{total} (%)
Low	120.5	7.7	6.4	118.8	6.3	4.7	8.0
Medium	249.9	12.7	5.2	260.6	14.7	5.6	7.6
High	499.5	7.9	1.6	469.9	31.5	6.9	7.1

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CHAPTER 5

Discussion

To the best of our knowledge, this is the first study using a commercial ELISA to measure the peripheral serum IGF-1 concentration, which is found to relate to sperm parameters in elephants. Our analyses reveal a significantly positive correlation between serum IGF-1 concentration and the proportion of spermatozoa with an intact acrosome in Asian elephants. The previous studies have validated that the IGF-1 receptor is primarily localized to the acrosomal region of the bovine and human sperm (Henricks et al., 1998; Naz and Padman, 1999), suggesting this region is involved in capacitation and the acrosome reaction. Moreover, in buffaloes, supplementation of IGF-1 in semen has a positive effect on maintaining the quality of sperm acrosomal membrane (Selvaraju et al., 2010). Furthermore, detecting the acrosomal status of sperm helps improve the fertility rate. Abnormality of acrosomal morphology has been implicated in compromised fertility in other species, including boar during artificial insemination (AI) procedure (Tardif et al., 1999) and human during *in vitro* fertilization (IVF) process (Menkveld et al., 1996). Furthermore, stallions with higher seminal IGF-1 concentrations resulted in greater pregnancy rates (Novak et al., 2010). These results suggest that increased serum IGF-1 concentration may be related to improved sperm quality.

Present results revealed a positive correlation between IGF-1 level and normal sperm head morphology. This observation is consistent with a recent study in men, demonstrating a significantly lesser concentration of IGF-1 is associated with the morphologically abnormal sperm head (Lee et al., 2016). Also, the amount of seminal plasma IGF-1 is positively correlated with the percentage of morphologically normal spermatozoa (Glander et al., 1996). An unexpected finding of our study was that repeated ejaculates increased the percentage of normal sperm parameters

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(including sperm head morphology, motility and viability, etc.) with greater IGF-1 levels in every individual bull. It was suggested previously that a reduction in head abnormality of spermatozoa can be observed after frequent stimulations, probably because of the accumulation of senescent semen in Asian elephants (Imrat et al., 2014). Thus, we could assume that repeated semen collections can improve the head abnormality of spermatozoa with IGF-1 level. However, the underlying reasons for the improvement of sperm head morphology together with IGF-1 concentrations are unclear.

Our findings identified a significantly positive correlation between IGF-1 concentration and testosterone level. The present results agreed with the previous investigations indicating that the circulating serum IGF-1 concentration is associated with serum testosterone level in other species (Ditchkoff et al., 2001; Brito et al., 2007). Testosterone production is not only regulated by the endocrine system, and it also depends upon local paracrine and autocrine factors such as IGF-1 (Gelber, 1992; Yoon and Roser, 2011). Moreover, testosterone can facilitate spermatogenesis as a paracrine factor in the Sertoli cells (Meeker et al., 2007). IGFs have been suggested to be the most important growth factor in regulating the developments and functions of testis and the number of Sertoli cells (Pitetti, 2013; Griffeth et al., 2014). In testis, testosterone and IGF-1 are secreted from Leydig cells. In addition, it has been shown that testosterone significantly increases the productions of IGF-1 and IGF-1 binding proteins (IGFBPs) on Leydig cells by a complex mechanism (Ashton et al., 1995).

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Large variations in serum IGF-1 concentration were observed among seven elephant bulls, possibly because of nutrition (Brito et al., 2007; Selvaraju et al., 2012; Tran et al., 2016). In the current study, all of the bulls were equally classified as having the same nutritional status (body condition scores: 3.0–3.5). Moreover, the same feeding protocol has been maintained with the foods provided at 6.0% of the body weight per day. However, all bulls were chained separately in the forest for

foraging in the afternoon. Thus, the availability (quantity and quality) of foods might vary upon each grazing.

In addition to nutrition, several factors such as age, IGFBPs and other hormones may contribute to these variations. For example, IGF-1 in testes and blood has been investigated to correlate with ages, in particular, the onset of puberty; the strong contents of IGF-1 and its receptors are shown in Leydig cells and spermatogonia, and plasma concentration of IGF-1 peaks during puberty age in male horses (Hess and Roser, 2001; Yoon et al., 2011). Furthermore, we also identified the age-associated semen quality in Asian elephants, which was similar to the previous study; the highest semen quality and serum testosterone were found in the 23–43-year-old age group, whereas the quality was the lowest at age 10 to 19 years (Thongtip et al., 2008). Puberty of captive elephants was defined as 10–15 years of age (Schulte, 2006). Thus, these could explain why E1 aged puberty was observed a high level of serum IGF-1 with the poorest semen quality whereas an elderly elephant (E4) aged 45 years old showed a great percentage of normal sperm parameters (> 60%) but with the lowest concentrations of IGF-1 and testosterone, therefore, which may cause the absence of significances between IGF-1 and other variables. Besides, IGFBPs have been suggested to regulate the reproductive cells by inhibiting or enhancing IGFs productions (Le Gac et al., 1996; Naz and Padman, 1999; Yoon et al., 2011; Yoon et al., 2015). On the other hand, IGF-1 is under the controls of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH and IGF-1 pathways are intimately connected (Cannarella et al., 2018). Additionally, IGF-1 productions are mediated and controlled by growth hormone (GH) in male fertility (Chandrashekar et al., 1999). Gonadal functions are influenced by the actions of GH and IGF-1 on pituitary gonadotrophs and gonadal sites (Choubey, 2020). Thus, factors influencing the productions of gonadotrophins and GH may also affect IGF-1 concentration.

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Despite the variability, the mean value of IGF-1 level we obtained in the present study (mean 326.3 ng/mL, range 167.4 to 542.7 ng/mL) was within the range of the only report previously determined serum IGF-1 of male Asian elephants (mean 307 ng/mL) using a radioimmunoassay (RIA) (Govoni et al., 2011). ELISA is considered as a relatively safe and easy method to operate compared to RIA. In the current study, the acceptable R^2 value together with an overall acceptable recovery rate of serial dilutions suggests a high accuracy in the linearity test. The inter-assay (< 10%), intra-assay (< 10%) and total imprecision CV (< 8.5%) indicate high precision. Thus, we could confirm that this ELISA can be used for analyzing circulating IGF-1 concentration in the elephant in terms of the acceptable results of accuracy and precision.

The present results are in accordance with these findings in which a higher serum IGF-1 level contributes to a positive effect on male fertility; however, the values for IGF-1 are varied among species. For example, as demonstrated in men, the IGF-1 level with normal semen parameters is estimated at 175.4 ± 42.7 ng/mL, whereas the IGF-1 level with abnormal sperm parameters is lesser (137.6 ± 27.1 ng/mL) (Lee et al., 2016). During the breeding season, the mean level of serum IGF-1 is the greatest (63.6 ng/mL) ranging from 5.81 to 224.9 ng/mL in the male white-tailed deers (Ditchkoff et al., 2001). Also, the IGF-1 value for buffalo bull is much higher than men, deer and elephants with a mean concentration of 1555.22 ng/mL (range 927 to 2247 ng/mL) is positively correlated with semen mass motility and sperm concentration. In Thailand, the low fertility rate (only 1.8–2% a year) of Asian elephants has been detected (Thitaram, 2011). This trend may be helpful to exclude poor sperm when performing AI and then minimize low fertility or pregnancy failure in elephants.

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5.1 Conclusions

The results of the present study indicate the correlation of greater circulating IGF-1 concentration and the sperm acrosome integrity and head normality, suggesting the increased IGF-1 level may be related to good sperm quality in Asian elephants. Unexpectedly, repeated semen collections by transrectal massage coincided with greater IGF-1 concentrations and higher percentages of normal semen variables has been demonstrated. However, the potential role of IGF-1 in the spermatogenesis or semen production of elephant bulls needs to be elucidated because testosterone has a positive effect on IGF-1 concentration.

5.2 Limitation and suggestion of this study

In the present study, we failed to detect seminal plasma IGF-1 even though we used the same ELISA kit and protocol with the detection of serum IGF-1. We did find IGF-1 in the seminal plasma, but the concentration is lower than the minimum detection range of the ELISA kit. Thus, it is not appropriate to do the ELISA validation process. Based on that, we speculate some factors which contribute to the failure of this experiment:

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1. Duration of sample storage is approximately half-year

   The IGF-1 probably degraded due to the long duration of storage at -20 ℃. Thus, it is vital to complete the experiment as soon as possible. Besides, thawing samples several times is not appropriate.

2. Concentration of seminal IGF-1 is lower than ELISA minimal detection range

   The extraction process is necessary for concentrating the IGF-1 level.

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