การแช่แข็งน้ำเชื้อ การผสมเทียมด้วยการส่องกล้องและการย้ายฝากตัวอ่อนแพะในประเทศไทย

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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SEMEN CRYOPRESERVATION, LAPAROSCOPIC ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER IN GOAT IN THAILAND

Miss Nitira Anakkul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
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นิธิรา อนัคกุล: การแช่แข็งน้ำเชื้อ การผสมเทียมด้วยการส่องกล้องและการย้ายฝากตัวอ่อนแพะในประเทศไทย (SEMEN CRYOPRESERVATION, LAPAROSCOPIC ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER IN GOAT IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร. มงคล เตชะกำพุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.น.สพ.ดร. ธีรวัฒน์ ธาราศานิต, ดร. เด็บบรา เค เบิร์ก, 120 หน้า

จุดประสงค์ของการศึกษาเพื่อพัฒนาการแช่แข็งน้ำเชื้อ การผสมเทียมด้วยวิธีส่องกล้องลาพาโรสโคป และการย้ายฝากตัวอ่อนในแพะเพื่อเพิ่มประสิทธิภาพด้านการสืบพันธุ์และการปรับปรุงพันธุ์ โดยประกอบด้วย 6 การทดลอง

การทดลองที่ 1, 2 และ 3 ศึกษาผลของอีเควิกซ์ เอสทีเอ็ม เพส และกลีเซอรอลที่ใส่ในสารละลายเจือจางน้ำเชื้อสำหรับแช่แข็งต่อความสามารถในการแช่แข็งและความสามารถในการปฏิสนธิ ทำการตรวจประเมินคุณภาพน้ำเชื้อ ได้แก่ อัตราการเคลื่อนที่ของตัวอสุจิ อัตราส่วนตัวอสุจิมีชีวิต ความสมบูรณ์ของอะโครโซม ความสมบูรณ์ของเยื่อหุ้มเซลล์อสุจิ และความสมบูรณ์ของสารพันธุกรรม ก่อนและหลังการแช่แข็ง และตรวจการเคลื่อนที่ของอสุจิโดยการใช้เครื่องตรวจวิเคราะห์อสุจิด้วยคอมพิวเตอร์ภายหลังการเก็บรักษาที่ 37°ซ ที่ 0, 1, 2 และ 3 ชั่วโมง ผลการศึกษาพบว่าความเข้มข้นของกลีเซอรอลที่ระดับ 10% และอีเควิก เอสทีเอ็ม เพส ที่ระดับ 0.5% สามารถป้องกันตัวอสุจิจากความเย็นที่เกิดขึ้นระหว่างกระบวนการแช่แข็งและการละลาย รวมถึงให้อัตราการตั้งท้องในระดับที่ยอมรับได้

การทดลองที่ 4 ศึกษาผลของฤดูกาลต่อลักษณะของน้ำเชื้อและความสามารถในการแช่แข็งในแพะพันธุ์ลูกผสมซาเนนสายเลือด 75% ที่อยู่ภายใต้สภาพอากาศแบบร้อนชื้น (ที่ละติจูด 18 องศาเหนือ และลองจิจูด 100 องศาตะวันออก) โดยน้ำเชื้อสดที่รีดเก็บมีคุณภาพสูงในฤดูร้อนและฤดูหนาว ส่วนคุณภาพน้ำเชื้อภายหลังการละลายในฤดูหนาวมีรูปแบบการเคลื่อนที่ของอสุจิ และความสมบูรณ์ของเยื่อหุ้มเซลล์อสุจิสูงกว่าฤดูอื่นอย่างมีนัยสำคัญ อย่างไรก็ตามคุณภาพน้ำเชื้อทั้งสดและแช่แข็งมีคุณภาพเพียงพอสำหรับการนำไปใช้ในการผสมเทียมได้ตลอดทั้งปี

การทดลองที่ 5 ศึกษาการเคลื่อนที่ของตัวอสุจิแช่แข็งภายหลังการผสมเทียมด้วยเทคนิคส่องกล้องลาพาโรสโคป และเปรียบเทียบผลของปริมาณตัวอสุจิและตำแหน่งการปล่อยน้ำเชื้อในปีกมดลูกข้างเดียวหรือทั้งสองข้างต่ออัตราการตั้งท้อง ย้อมสีตัวอสุจิด้วยสีเซลล์แทร็คเกอร์ กรีน (สีเขียว) หรือเซลล์แทร็คเกอร์ เร้ด (สีแดง) เพื่อตรวจการกระจายตัวของอสุจิจากชิ้นส่วนมดลูกและท่อนำไข่ที่ได้จากการผ่าตัดเก็บมดลูกและรังไข่ หลังปล่อยน้ำเชื้อที่ย้อมสีเขียวเข้าที่ปีกมดลูกข้างซ้าย และสีแดงเข้าที่ปีกมดลูกข้างขวา พบว่าสามารถตรวจพบอสุจิที่ถูกปล่อยที่ปีกมดลูกข้างเดียวได้ในปีกมดลูกทั้งสองข้าง และทำการผสมเทียมด้วยเทคนิคส่องกล้องลาพาโรสโคปด้วยน้ำเชื้อแช่แข็งจำนวน (60 และ 120 ล้าน) และตำแหน่งที่ต่างกัน (1 หรือ 2 ข้าง) อัตราการผสมติดไม่มีความแตกต่างอย่างมีนัยสำคัญระหว่างกลุ่ม ดังนั้นการผสมเทียมที่ปีกมดลูกเพียงข้างเดียวด้วยน้ำเชื้อแช่แข็งจำนวน 60 ล้านตัว เพียงพอต่อการผสมเทียมในแพะ

การทดลองที่ 6 นำเอาการผสมเทียมด้วยเทคนิคส่องกล้องลาพาโรสโคปและการย้ายฝากตัวอ่อน มาใช้ในการผสมข้ามพันธุ์เพื่อผลิตแพะสีดำ การทดลองที่ 1 ทำการผสมเทียมด้วยเทคนิคส่องกล้องลาพาโรสโคปโดยใช้น้ำเชื้อแช่แข็งจากพ่อแพะสีดำพันธุ์ออสเตรเลียนเมลานกับแพะสีขาวพันธุ์ซาเนน (75%) จำนวน 70 ตัว แพะตั้งท้อง 50 ตัว ได้ลูกแพะจำนวนรวม 68 ตัว โดยเป็นลูกแพะสีดำ 10.29% สีขาว 39.71 % และสีอื่น ๆ 50% ในการทดลองที่ 2 ทำการศึกษาการผสมข้ามพันธุ์ใน 2 รูปแบบ คือ รูปแบบแรกทำการผสมเทียมแพะเพศเมียพันธุ์แบล็คเบงกอลจำนวน 7 ตัว ด้วยน้ำเชื้อแช่แข็งจากพ่อพันธุ์ออสเตรเลียนเมลาน และรูปแบบที่สองทำการผสมเทียมแพะเพศเมียสีดำซึ่งเป็นลูกผสมออสเตรเลียนเมลาน (50%) จำนวน 7 ตัว ด้วยน้ำเชื้อแช่แข็งจากพ่อพันธุ์แบล็คเบงกอล ตัวอ่อนทั้งสิ้นจำนวน 30 ใบ ที่เก็บได้จากการผ่าตัด อยู่ในระยะ 4 ถึง 8 เซลล์ (อายุ 3 วัน) อัตราการตั้งท้องจากการย้ายฝากไปยังแพะตัวรับจำนวน 30 ตัว เท่ากับร้อยละ 30 ลูกแพะทั้งหมด 9 ตัว ที่ผลิตได้จากทั้งสองรูปแบบการผสมมีสีดำ มีน้ำหนักแรกเกิดเฉลี่ย 2.56±0.95 กิโลกรัม สามารถสรุปได้ว่าการนำเอาการผสมเทียมด้วยเทคนิคส่องกล้องลาพาโรสโคปมาใช้ร่วมกับการย้ายฝากตัวอ่อนประสบความสำเร็จในการผลิตและรักษาศักยภาพทางพันธุกรรมในการรักษาลักษณะการแสดงออกของสีดำ

โดยสรุปพบว่าการปรับปรุงการแช่แข็งน้ำเชื้อโดยการเพิ่มสารเคมีในสารละลายน้ำเชื้อ การศึกษาผลของฤดูกาลในการเก็บและแช่แข็งน้ำเชื้อ รวมทั้งการผสมเทียมผ่านกล้องลาพาโรสโคปและการย้ายฝากตัวอ่อน สามารถนำไปใช้ในอุตสาหกรรมการเลี้ยงแพะโดยได้อัตราการตั้งท้องที่เหมาะสม เป็นวิธีการช่วยในการปรับปรุงพันธุ์แพะต่อไป

ภาควิชา.....สูติศาสตร์ เธนุเวชวิทยาและวิยาการสืบพันธุ์....
สาขาวิชา...วิทยาการสืบพันธุ์สัตว์ .....
ปีการศึกษา...2555.....

ลายมือชื่อนิสิต.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS: SEMEN CRYOPRESERVATION / EQUEX STM PASTE / SEASON / SPERM DISTRIBUTION / LAPAROSCOPIC ARTIFICIAL INSEMINATION / EMBRYO TRANSFER / GOAT

NITIRA ANAKKUL: SEMEN CRYOPRESERVATION, LAPAROSCOPIC ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER IN GOAT IN THAILAND. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., DOCTORAT DE 3e CYCLE. CO-ADVISOR: AISST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., DEBRA K BERG, Ph.D., 120 pp.

The study is aimed to do the research on semen cryopreservation, laparoscopic artificial insemination and embryo transfer in goats and provide valuable practical opportunities to improve reproductive efficiency and to enhance the genetic improvement. It composed of six experiments.

Experiment I, II and III aimed at evaluation the effect of Equex STM Paste and glycerol supplemented in freezing extender on freezability and fertilizing ability. The semen qualities including motility, viability, morphology, acrosome integrity, membrane functional integrity and DNA integrity, were evaluated before processing and after cryopreservation. Computer-assisted-semen analysis (CASA) was used to characterize the sperm motion patterns during the thermal resistance test at 0, 1, 2 and 3 h post-thawing. The freezing medium containing 10% glycerol and 0.5% Equex STM Paste sufficiently protected goat spermatozoa against cryoinjury during freezing and thawing, and provided an acceptable pregnancy rate.

Experiment IV, aimed to study the seasonal effect on semeinal characteristics and freezability of crossbred Saanen bucks. Overall, the fresh semen collected in summer and winter provided a higher in quality. The post-thaw semen qualities were not significant different except for motility characteristics and plasma membrane integrity which showing their best in winter. However, the quality of semen collected and frozen throughout the year was acceptable for AI.

Experiment V, aimed to evaluate the distribution of frozen-thawed spermatozoa after laparoscopic artificial insemination (LAI) and to compare the effect of sperm numbers and deposition sites (unilateral and bilateral sites) on pregnancy rate. The post-thaw spermatozoa were stained with CellTracker™ Green CMFDA (CT-Green) or CellTracker™ Red CMPTX (CT-Red), and then were deposited into the left and right uterine horns, respectively. The sperm were collected separately from each side of uterus and oviduct after an ovariohysterectomy. Spermatozoa deposited into only one uterine horn distributed to both ipsilateral and contralateral sides. The LAI was performed in 60 does using different numbers of spermatozoa (60 and 120 $x10^6$ sperm) and deposition sites (unilateral or bilateral). The pregnancy rates were not significantly different in does deposited with different doses or insemination sites ($P>0.05$). The unilateral insemination with 60 $x10^6$ sperm was sufficient for LAI in goat.

Experiment VI, aimed to use LAI and embryo transfer (ET) to produce crossbred black-colored goat. In Experiment 1, LAI with frozen-thawed semen of black buck (Australian Melaan) was performed in 75% Saanen crossbred does (white color, n=70). The total numbers of 68 kids were born from 50 does. The skin colors of kids born were black (10.29%), white (39.71%) and other colors (50%). In Experiment 2, two cross-breeding programs were tested including program I: frozen semen of Australian Melaan inseminated to Black Bengal female (n=7) and program II: frozen semen of Black Bengal inseminated to 50% Australian Melaan (n=7). Thirty embryos at 4-8 cell stage (day 3) were surgically collected and transferred into 30 recipients resulting 30% of pregnancy. Nine kids born from both protocols were black in color with 2.56±0.95 kg birth weights. LAI and ET can be successfully combined to produce and sustain the genetic potential encoding the black-skin color.

In conclusion, by an improvement of semen freezing via the semen extender, a study of seasonal effect on semen collection and freezabitity as well as the practical techniques for LAI and ET, can be applied to a goat industry for genetic improvement with an acceptable pregnancy rate.

Department: Obstetrics, Gynaecology and Reproduction Student's Signature .....
Field of Study: Theriogenology Advisor's Signature .....
Academic Year: 2012 Co-advisor's Signature .....
Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

AI	artificial insemination
ALH	amplitude of lateral head displacement
AM	Australian Melaan
AO	Acridine orange
BB	Black Bengal
BCF	beat cross frequency
°C	Celsius
CASA	computer-assisted-semen analysis
CAE	Caprine Arthritis Encephalitis
CH	corpus haemorrhagicum
CL	corpora lutea
cm	centimeter
CO_2	carbon dioxide
CT-Green	CellTracker™ Green CMFDA
CT-Red	CellTracker™ Red CMPTX
DNA	deoxyribonucleic acid
°E	longitude is east
E	Equex STM Paste
ET	embryo transfer
Exp.	experiment
g	gravity
GLM	general linear model
h	hour
hCG	human chorionic gonadotropin
HOST	hypo-osmotic swelling test
Hz	hertz
IVF	*in vitro* fertilization
LAI	laparoscopic artificial insemination

LIN	linearity
LSD	least significant different test
FITC-PNA	fluorescein isothiocyanated peanut agglutinin
mg/kg	milligram/kilogram
min	minute
ml	milliliter
mM	millimolar
mOsm/kg	milliosmolar/kilogram
MOT	motility
°N	latitude is north
NA	not applicable
OVH	ovariohysterectomy
OH	hydroxyl
PBS	phosphate buffer saline
PI	propidium iodide
PMOT	progressive motility
PMSG	pregnant mare serum gonadotropin
sHOST	swollen tail in hypo-osmotic swelling test
sec	second
SE	standard error
SD	standard deviation
SDS	sodium dodecyl (lauryl) sulphate
STR	straightness
UTJ	utero-tubular junction
TCF	Tris (hydroxymethy laminomethane)- citric acid-fructose
v/v	volume/volume
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity

w/v	weight/volume
w/w	weight/weight
µg/ml	microgram/milliliter
µl	microliter
µM	micromolar
µm/s	micrometer/second

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Goat production in Thailand is important to the industry and demand is increasing especially in the southern part of Thailand. Goats (*Capra hircus*) can utilize low quality roughage for meat and milk production and they possess a high rate of reproductive efficiency. Meanwhile, research on reproductive biotechnology in the goat in Thailand is still limited compared with pig and cattle. Artificial insemination (AI) and/or embryo transfer (ET), which allows the production of more offspring from high genetic resources, are studied but an application of these technologies to the goat industry is limited. This is due to a lower success compared to natural breeding. However, these techniques can be used to propagate the disease free animals especially the two major diseases that affect reproduction and health, Brucellosis and Caprine Arthritis Encephalitis (CAE). Both of these are highly contagious within goat herds.

AI using frozen-thawed semen plays a major role in disseminating superior genetics thoughout the goat industry rapidly improving genetic gain; however, freezing induces detrimental effects to spermatozoa viability. An improvement of a quality of frozen–thawed semen is one of the targets for goat AI. Several strategies have currently been used to improve membrane fluidity of the spermatozoa such as modifying the sperm plasma membrane with cholesterol-loaded-cyclodextrin (Klein et al., 1995; Zahn et al., 2002; Farshad et al., 2011) and also using some detergents to alter the interactions between lipoproteins in the egg yolk and plasma membrane resulting in a more accessible lipoproteins to the sperm plasma membrane (Amann and Pickett, 1987). Sodium dodecyl sulphate (SDS) and Equex STM Paste is a water-soluble anionic detergent that has been supplemented as an additive in the freezing extender (Dewit et al., 2000). The positive effects of Equex STM Paste have been shown especially when egg yolk was presented in the freezing extender (Pursel et al., 1978; Dewit et al., 2000; Aboagla and Terada, 2004[a]). Although the certain amount of SDS (active ingredient of

Equex STM Paste) and Equex STM Paste has not been clearly identified (Peña et al., 2003), they have been demonstrated to be beneficial for goat semen cryopreservation. (Aboagla and Terada, 2003; 2004[a,b]; Chang et al., 2006; El-Kon et al., 2010; Bittencourt et al., 2008). Therefore, the optimal concentration of Equex STM Paste, which beneficial for frozen-thawed goat spermatozoa, remains to be studied.

In order to get an achievement of AI program, semen should be collected in an appropriate time with a proper management of semen storage and use (Leboeuf et al., 2000). Although spermatogenesis in the male goat can occur throughout the year, the qualities and quantities of semen are influenced by various factors (Mohri et al., 1970). Season of the year seems to be an important factor that have more effect on semen quality especially in higher latitudes (higher than 35°N) (Corteel, 1977; Leboeuf et al., 2000; Barkawi et al., 2005), which the decreasing of photoperiod stimulates reproductive activity (Chemineau et al., 1987; Janett et al., 2003). The effects of seasonal variation on freezability and fertility of semen collected are disagreeing (Leboeuf et al., 2000). Thailand is located in the tropical part with hot weather in most of the year. Then, collection the semen in appropriate time results in getting the better semen quality. However, there are less information about goat semen production and freezability in Thailand.

A low success of AI in goat is elsewhere reported due to a difficulty to pass through the AI catheter into cervical canal. This makes AI not so popular in small ruminant as in cattle. To achieve a good pregnancy rate, semen should be deposited directly into the uterine horn. Laparoscopic artificial insemination (LAI) is a technique allows depositing semen directly into the uterine horns (Baldassarre and Karatzas, 2004). LAI has been shown to increase the conception and kidding rates (Epstein and Herz, 1964). Nevertheless, there is only few report compared to sheep (Martínez-Rojero et al., 2007). Moreover, the information on the number of spermatozoa and the uni- or bilateral insemination to produce a good pregnancy rate is still unclear.

Embryo transfer (ET) is the second generation reproductive biotechnology for high genetic female distribution and helping to produce specific desired breeds such as

black goat which are in demand and more expensive compared to other colors. This is due to the fact that white, brown and tan colors are dominant over black color (Asdell and Buchanan Smith 1926; Adalsteinsson et al., 1994). The skin color is genetically controlled by the presence of two subtypes of melanin pigments (eumelanin and phaeomelanin). The eumelanin represents black color while the other pigment typically expresses reddish brown or tan colors. Although melanocytes can produce both types of pigments, eumelanin is genetically recessive. Therefore, when black goats are mated with other colored goats, coat colors of offspring are remarkably variable (Asdell and Buchanan Smith, 1926). ET can be used as a tool to produce a number of offspring from black goat, by collecting embryos and transfer to recipients.

The content of research work (Fig. 1) is aimed to do the research on semen cryopreservation, LAI and ET in goats and provide valuable practical opportunities to improve reproductive efficiency and to enhance the genetic improvement for goat industry.

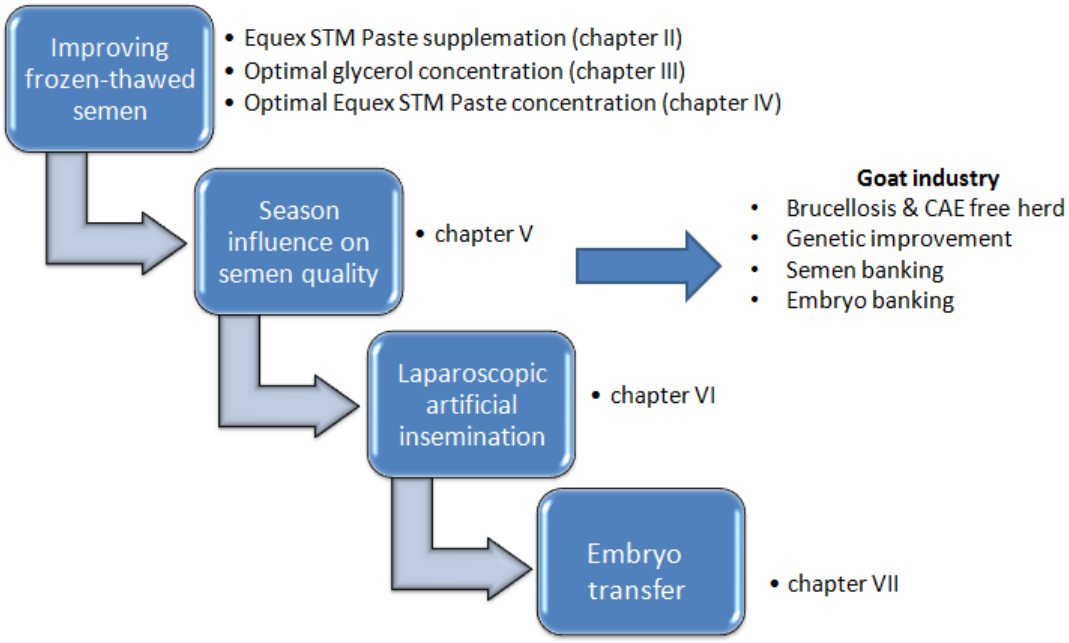

Figure 1 The framework of thesis which composes of six experiments.

1.2 Literature review

Nowadays, new biotechnologies have been developed in agriculture. These techniques are being used for the disease prevention, improvement, and preservation of livestock genetics and the enhancement of reproductive efficiency. The application of reproductive biotechnology in goat supports the increasing of genetic progress. These technologies such as AI, ET which allow the animals that have high genetic merit, to produce more offsprings than natural breeding. Some of these techniques in combination with hormonal synchronization of estrus and ovulation in seasonally reproductive species such as goat allow the production of kids and milk out of breeding season (Corteel et al., 1988; Chemineau and Cognié, 1991).

1.2.1 Preservation of goat semen

Cryopreservation is a technique for storage goat semen using for AI. Many diluents have been examined for freezing goat semen such as tris–yolk, sodium citrate–glucose–yolk, reconstituted skim cow milk, lactose–yolk and raffinose-yolk (Leboeuf et al., 2000). The most widely used media seem to be reconstituted skim cow milk-glucose (Corteel, 1974) and tris–glucose–citric acid–yolk (Salamon and Ritar, 1982). More recently, commercial extenders with no biological components have been developed to improve sanitary safety in semen processing (Gill et al., 2003).

General consideration in goat semen cryopreservation is the detrimental of seminal plasma on the viability of the spermatozoa in diluents containing egg yolk or milk-based medium. Bulbourethral glands of goat secretes specific enzymes in seminal plasma, which catalyse the hydrolysation of egg yolk lecithin (Pellicer-Rubio et al., 1997) and milk triglycerides of the extender (Chemineau et al., 1999). These processes release lysolecithin and fatty acids (Iritani and Nishikawa, 1961; 1963) which are toxic for sperm (Roy, 1957; Iritiani et al., 1961; Nunes et al., 1982). This has led to remove of seminal plasma by centrifugation to protect the sperm. Many researchers have been reported about the advantages in seminal plasma removal (Ritar and Salamon, 1982). However, it should be noted that washing by centrifugation is a complex and time-consuming process, and also causes some loss of spermatozoa (Corteel, 1981). Other investigators considered the membrane damage by centrifugation to be more

detrimental than that caused by enzyme. They recommended to process goat semen in the same way as bovine semen (Evans and Maxwell, 1987; Tuli and Holtz, 1994; Azerêdo et al., 2001) or use of 1.5% egg yolk in a tris-based diluent without removal of seminal plasma (Ritar and Salamon., 1982).

Semen cryopreservation has many advantages but freezing and thawing causes detrimental effects to sperm. In relation to this, various methods for processing and freezing have been developed to reduce cryogenic injures to spermatozoa. A cryoprotectant is included in a freezing medium to minimize the physical and chemical stresses resulting from the cooling, freezing, and thawing processes. Glycerol is a widely used as a membrane–permeable cryoprotectant whereas most common non-penetrating cryoprotectant used is egg yolk (Ritar and Salamon, 1982; Tuli and Holtz, 1994). Several cryoprotocols have been developed for freezing goat spermatozoa. Addition of a surfactant, for example sodium dodecyl sulphate (SDS), Equex STM Paste and Orvus ES paste, to a Tris extender has been reported to improve post-thaw quality of spermatozoa in many species such as dog (Rota et al., 1997; Peña and Linde-Forsberg, 2000), pig (Pursel et al., 1978; Buranaumnuay et al., 2009), bull (Arriola and Foote, 1987), horse (Martin et al., 1979), deer (Cheng et al., 2004) and sheep (Akourki et al., 2004). In goat, there are some reports about addition of SDS but not Equex STM Paste. SDS is an active compound in Equex STM Paste. It can solubilize egg yolk lipids in the freezing extender, which had beneficial effects on post thawing goat semen (Aboagla and Terada, 2004[a;b]).

Diluted buck semen is cooled to 4 to 5°C about 1.5 to 4 h and then frozen as pellets or straws, placed on a rack, and frozen in liquid nitrogen vapor (4 to 5 cm above liquid nitrogen). Both styrofoam box containing liquid nitrogen and a programmable freezer that controls the cooling rate, have been successfully used for cryopreservation goat semen.

1.2.2 Seasonal effect on semen production

The seasonal nature of sexual activity of many mammals is well recognized (Eric and Herman, 1944). In ungulates such as sheep and deer, the male reproductive system is under the photoperiodic control. (Lincoln, 1978; Mirarchi et al., 1978; Lincoln and Kay, 1979; Karsch et al., 1984; Sandford et al., 1984; Asher et al., 1989). The increasing of melatonin, which secreted from the pineal gland during the dark hours, is responsible for the transition to the breeding season (Fitzgerald, 1997). In goat, many factors influences on sexual behavior and semen quality such as breed, geographical location, season of the year, testicular size and circulating gonadotropins. However, season of the year seems to have more effect on semen quality (Barkawi et al., 2005). The seasonal factors have an overriding impact on reproduction, especially in higher latitudes which sexual behavior, testicular size and sperm production are influenced by photoperiodic changes (Ortavant, 1977; Laubser et al., 1982). Different from horses, decreasing daylength stimulates reproductive activity in goat (Chemineau et al., 1987; Janett et al., 2003). In latitudes above 40°N, the variations in seminal characteristics is very marked (Corteel, 1977) and sperm production increases significantly when day length is decreasing. The goats in mid and high latitudes (higher than 35°) show marked seasonality (Leboeuf et al., 2000). Their breeding season begins at the end of summer and continues through autumn and winter, whereas goats in the tropics or subtropics may breed at all times of the year or show trends in kidding related to environmental factors other than photoperiod (Chemineau and Xande, 1982; Restall, 1991). The length of the breeding season is increasing when the latitude is decreasing.

In subtropical and tropical environment, nutrition appears to be the major modulator of sexual activity in small ruminants (Gebre, 2007). Moreover, a temperature may affect on the reproductive ability. The volume of semen and concentration of spermatozoa were found to be decreased in high temperature (Hiroe and Tomizuka, 1966; Yokoki and Ogasa, 1977), and by high relative humidity and rainfall in Indian goats (Mukherjee et al., 1953).

In Barf, Iran (latitude of 29° 37'), testicular size, semen quality and quantity were significantly higher during the summer months (Zamari and Heidari, 2006). Similar to British (Ahmad and Noakes, 1995), Damascus (Al-Ghalban et al., 2004) and Zaraibi goats (Barkawi et al., 2005), mean scrotal circumference was largest in summer. Although, the variation in testis size can be explained by changes in body, seasonality may have a greater influence on testis size than body weight (Ahmad and Noakes, 1995; Delgadillo et al., 1991).

The quality of the spermatozoa is affected by season. The percentage of motile spermatozoa was found to be high during the sexual season and low outside this period. In the tropics where the influence of photoperiod is minimal, semen quality is usually higher when collected in the rainy than in the dry season. This shows a positive influence of nutrition, because rainy season feeds have more nutrients than feeds in dry season (Carmenate and Gamcik, 1982). The percentage of abnormal morphology spermatozoa was 5 to 8% during the sexual season and 10 to 18% at other times (Corteel, 1977; Tuli and Holtz, 1992). The volume of the ejaculate of Alpine and Poitevine breeds is high in breeding season while decreases to a minimum in nonbreeding season. However; the concentration of the ejaculate are converse because the accessory glands are active in the breeding season due to the high concentration of testosterone (Corteel, 1977). The low level of circulating plasma testosterone and a reduction in the thickness of the seminiferous tubules, which due to the lower number of spermatic layers, brought about the low libido and semen quality of Zaraibi bucks in spring (Barkawi et al., 2006). On the other hand, the number of spermatic layers, which were the best indicators of testicular activity for spermatogenesis process, was highest in autumn (Barkawi et al., 2006).

The effects of seasonal variation on freezability and fertility of semen collected in and out of breeding season are disagreeing. Some investigators found that freezability of semen and fertility of frozen-thawed spermatozoa was better when collected in the breeding season (Muhuyi et al., 1992). Moreover, both cooled and frozen-thawed spermatozoa obtained outside the breeding season provided significantly lower kidding

rate than semen processed in the breeding season (Corteel et al., 1980; 1988). Overall, for long-term storage, the semen of desired bucks should be collected and processed during the normal breeding season. Furthermore, Barkawi et al. (2006) had recommended not using the semen collected during the winter and spring for artificial insemination, since a lower in volume and qualities. However, no seasonal difference in fertility was also reported (Peskovatskov et al., 1974; Summermatter and Flukiger, 1982).

1.2.3 Artificial insemination (AI) and Laparoscopic artificial insemination (LAI)

AI is the most commonly applied means of reproductive biotechnologies in livestock (Holtz et al., 2008). The application of AI has made the contribution to genetic improvement programs. Because the development of AI based on semen preservation from desired male, bucks with the highest genetic merit have been selected. Preserved semen plays a major role in distribution of genetic, especially when using frozen semen for AI. This allows rapid and widespread scattering of improved genotypes and the changing of genotypes without transmitting diseases (Leboeuf et al., 2000), which facilitates international exchange of genetic material causing genetic diversity in goat. Nevertheless, AI in small ruminants is more difficult than in cattle because of a small body size and the complex anatomy of the cervical canal, the insemination into the uterus is then difficult. These results in a low conception rate, only 20 to 30% of pregnancy rates were reported (Sohnrey and Holtz, 2005).

Intrauterine insemination gives a higher conception rate either fresh or post-thaw semen because the semen is deposited closer to the site of fertilization (Sohnrey and Holtz, 2005). LAI also has known as intrauterine insemination, by-passes the cervix, deposits semen directly into the uterine horns. It is a minimally invasive, minor surgical procedure that requires the expertise. With this technique, the reproductive tract could be investigated clearly through the abdominal cavity using laparoscope so that the semen can be deposited directly into the lumen. Does and ewes may be laparoscopically inseminated many times throughout their lives (Mobini et al., 2002). The LAI has been shown to increase the conception and kidding rates by as much as twice the rate of the cervical technique (Epstein and Herz, 1964). It is recommended that an

insemination prior to an ovulation provided a higher fertility than post-ovulation (Ritar et al., 1990[a]). The ovulation can be effectively controlled by injection of pregnant mare serum gonadotropin (PMSG) around the time of removal of intravaginal progesterone sponges (Ritar et al., 1984) or with controlled internal drug release (CIDR) devices (Ritar et al. 1989).

There were a few reports in the literatures related to intrauterine AI in goats using cryopreserved semen (Martínez-Rojero et al., 2007). Conception rates varied from 20 to 90% have been reported in sheep and goat (Mobini et al., 2002); 29.2% in Creole goats from Mexico (Amaro et al., 1997), 33.3% (Martínez-Rojero et al., 2007), 41%. (Goonewardene et al., 1996), 71.2% in Cashmere goats (Ritar et al., 1987), 0 to 40% in goats of different flocks in Argentina (Martínez-Rojero et al., 2007). The successful rates depend largely on the amount and motility of spermatozoa being inseminated (Rodriquez et al., 1988).

Another advantage of LAI is the low number of spermatozoa required to be deposited (Maxwell et al., 1984). However, some impairment may occur if too small number of frozen-thawed spermatozoa is deposited (Hunter et al., 1982) or if the deposition is performed in different parts (Maxwell et al., 1984). The doses as low as 0.5 x 10^6 motile spermatozoa could be obtained with lambing, with a linear increase in fertilization rate as the concentration of motile spermatozoa increased (Eppleston et al.; 1994). However, to get more than 50% of lambing rate, at least 20 x 10^6 motile spermatozoa were deposited (Maxwell, 1986). Another reports recommended 50 x 10^6 of motile sperm (Rodriquez et al., 1988; Eppleston and Maxwell, 1995; Buckrell, 1997). In goat, the numbers of spermatozoa used for LAI is still inconsistent and variable in goat. Buckrell (1997) recommended 20 x 10^6 motile spermatozoa as a desired number per doe. However, a variable from 5 to 200 x 10^6 spermatozoa have been inseminated (Ritar et al., 1990; Baril et al., 1996; Buckrell, 1997; Sohnrey and Holtz, 2005; Martínez-Rojero et al., 2007).

The number of uterine horns being inseminated and sites of insemination were also studied. The deposition of semen in the middle of the uterine horn (half way

between the UTJ and the bifurcation) provided a higher percentage of lambs born than those receiving semen in the tip (as close as possible to the UTJ) or bottom (Maxwell 1986). The information about the number of uterine horns inseminated is still unclear. There are less reported about these in goat. Most LAI protocol in goat obtained from sheep (Martínez-Rojero et al., 2007). Half the total number of motile spermatozoa was deposited into the lumen of each horn approximately mid-way between the uterine body and the UTJ junction (Ritar et al., 1990[a]). In sheep, the percentage of ewes lambing was higher in ewes inseminated in two uterine horns than one ipsilateral horn (Maxwell, 1986). In contrast, Killeen et al. (1982) found that non-return rates were lower when inseminated in a single uterine horn but without indication that the inseminated horn was ipsilateral to the current ovulation. Correa et al. (1994), Eppleston et al. (1994) and Perkins et al. (1996) reported that no effect between double and single horn (without regard to ovulation site) insemination. Perkins et al. (1996) mentioned that the ovarian manipulation may have adversely affected on pregnancy rates. Furthermore, compare with bilateral insemination, the unilateral insemination consumes lesser time and lesser manipulation to both uterine horns and abdominal viscera. The unilateral horn insemination was similar to that used during commercial sheep LAI (Perkins et al., 1996). In dog, the deposition of a 3-ml inseminate containing 300 $x10^6$ frozen–thawed spermatozoa resulted in a distribution through the entire uterine lumen, whether deposited at the tip of one horn or uterine body (Fukushima et al., 2010).

1.2.4 The coat color of black goat offspring

The pigments in hair and skin are made of the protein melanin. Melanin in goats consists of two main types: eumelanin and phaeomelanin. The skin color is genetically controlled by the presence of these two subtypes of melanin pigments. The eumelanin is predominantly produced from tyrosine and represents black color while the other pigment typically expresses reddish brown or tan color. Various genes control how much of each type of pigment is made and where it is deposited. Agouti is important color gene in mammals. It controls amount and distribution of phaeomelanin and eumelanin, so the variable of goat colour depends on the Agouti locus. At this locus,

phaeomelanin is dominant. The final color of the goat is due to the interaction of eumelanin and phaeomelanin. Moreover, Asdell and Smith (1926) suggested that brown is dominant to the black series, tan and red. Black is recessive to fawn. Due to the recessive of eumelanin which control black color, the mating between black and black got the offspring in black and chocolate. But, mating black with other color resulted in variable coat characteristics (Asdell and Smith, 1926).

The Australian Melaan is a black goat breed developed in Australia. The Australian Melaan takes its name from its color. The 'Mel' is from melanin or black pigment and the 'aan' is from Saanen. This breed is considered disease resistant and highly productive. Black Bengal goat or Bengal Black goat is the common name for a small breed of goat found in Bangladesh and Northeast India. The predominant coat color encountered is black. This breed is known for its adaptability, fertility, high productivity, skin softness and its excellence in production of quality meat (Amin et al., 2000). Husain et al. (1998) recommended that genetic improvement of the Black Bengal goat could be achieved by selection. Amin et al. (2000) also reported that cross breeding with Black Bengal goat can improve the carcass value. Cross-breeding has helped in increasing milk, meat and fibre production to a great extent.

1.2.5 Embryo transfer (ET) in goat

ET is a second generation reproductive biotechnology, after AI. Breeding programs using AI concentrates in the male to produce offspring, superovulation followed by embryo recovery and transfer is a successful method for distributing superior female genes (Isthwar and Memon, 1996). ET can be operated both in and out of breeding season but the best response is obtained when donor and recipient are cycling normally (Ishwa and Memon, 1996; Hill, 1996). The first embryo transfer in goat was reported by Warwick et al. (1934). After that, many researches involving embryo transfers in small ruminant have been done.

There are three methods of ET reported in small ruminants, namely, laparoscopic ET, surgical ET and transcervical ET (McKelvy et al., 1985). Laparoscopic ET was reported more successful (Ishwar and Memon, 1996). However, the success in ET is

also influenced from many factors such as management of donor and recipient does, estrus synchronization in donors and recipients, superovulation of donors, insemination technique, embryo collection, embryo evaluation and transfer of embryos (Ishwar and Memon, 1996). Superovulation of the donor is acquired by PMSG or follicular stimulating hormone (FSH). PMSG has a longer half-life (72 h) and required only a single dose 48 h before the progestagen removal, while FSH has a half-life about 6 h and is administered twice-daily beginning 48 h before the progesterone source is removed. Nevertheless, FSH provided better results in ovulation, fertilization rates and production of high quality embryos (Mobini et al., 2002). For insemination technique, LAI at 48 h after sponge removal provides high fertility and a low frequency of degenerated embryos (Trounson and Moore, 1974; Cognié, 1999; Cognié et al., 2003). There was variable information about stage of embryo for transfer. The differences of success may be explained by the site at which the transfers were made. However, it can be concluded that embryos recovered below the eight-cell stage should be transferred to the oviducts while embryos beyond eight cells should be transferred to the uterus (Armstrong and Evans, 1983). This was postulated that the uterus does not provide an environment suitable for the survival and development of two and four cells embryos (Averill and Rowson, 1958). The survival of embryos transferred as twins was significantly higher when both embryos were transferred to the same oviduct (unilateral transfer) (Armstrong et al., 1983).

1.3 Objectives

1. To compare the effect of different extenders supplemented with different glycerol concentrations and Equex STM Paste concentrations on frozen-thawed goat semen quality and fertilizing ability
2. To determine the quality and freezability of semen collected from crossbred Saanen born and raised in humid and tropical condition at different seasons of the year
3. To evaluate the distribution after intrauterine insemination with frozen-thawed spermatozoa labeled with fluorochrome
4. To study the effect of sperm numbers and insemination sites on pregnancy rates following the unilateral and bilateral laparoscopic insemination in goat.
5. To examine whether or not the production of black goat offspring using LAI, superovulation and ET techniques were efficiently performed

CHAPTER II

EFFECT OF EQUEX STM PASTE ON THE QUALITY AND MOTILITY CHARACTERISTICS OF POST THAWED CRYOPRESERVED GOAT SEMEN

2.1 Abstract

The aim of this study was to evaluate the effect of Equex STM Paste on goat semen quality after cryopreservation. Semen samples were consecutively collected once a week, from six mature bucks. The semen samples with adequate motility (>60%) and sperm concentration (>1,500x10^6 sperm/ml) were frozen in an egg yolk-Tris semen extender supplemented either with (E+) or without (E-) Equex STM Paste. The semen quality including motility, viability, morphology, acrosome integrity and membrane functional integrity were evaluated before processing and after cryopreservation. Computer-assisted-sperm analysis (CASA) was used to characterize the sperm motion patterns during the thermal resistance test at 0, 1, 2 and 3 h post-thawing. After thawing, the sperm motility was higher in E+ group at 0 ($P=0.0009$), 1 ($P>0.05$) and 2 h ($P>0.05$) compared to E- group. The positive effect of Equex STM Paste was also observed in terms of viability ($P=0.0002$), normal tail ($P<0.0001$) and the percentage of acrosome-intact spermatozoa ($P>0.05$). While semen in E- group exhibited better plasma membrane integrity, higher number of normal sperm head, some of the motility characteristics seem better than that of E+ group but not significantly. In conclusion, the improvement of the quality of post thawed buck semen in extender supplemented with Equex STM Paste was noticeable only in the first 2 h after thawing.

2.2 Introduction

Frozen semen is normally used in artificial insemination (AI) in goat; however, freezing and thawing reduce its semen qualities. An addition of cryoprotectant(s) in a freezing medium is necessary in order to minimize both physical and chemical stresses. Glycerol and egg yolk are commonly used as cryoprotectants in goat semen freezing

(Ritar and Salamon, 1982; Tuli and Holtz, 1994). Nevertherless, the diluents containing egg yolk cause detrimental effect on seminal plasma of the spermatozoa. This is due to the presence of egg yolk coagulating enzyme in the seminal plasma of goat (Iritani and Nishikawa, 1963), that why a removal of seminal plasma by centrifugation to protect the spermatozoa before cooling and freezing is necessary. The study on modifications of the diluents has been investigated (Purdy, 2006). Recently, an addition of Equex STM Paste to a Tris extender has been used to improve post-thaw quality of spermatozoa in many species. It exerts a beneficial effect on sperm quality of cryopreserved semen of dog (Rota et al., 1997; Peña and Linde-Forsberg, 2000), cat (Axnér et al, 2004), pig (Pursel et al., 1978; Buranaamnuay et al., 2009), cattle (Arriola and Foote, 1987), horse (Martin et al., 1979; Jimenez, 1987), deer (Cheng et al., 2004) and sheep (Akourki et al., 2004).

Addition of Equex STM Paste may also improve the freezability of goat semen. For the evaluation of semen quality, the sperm motility is one of the most important indicators (Kozdrowski, et al., 2007). The visual estimate is a common method; however, it is subjective depending on evaluators. A measurement of the percentage of motile sperm using computer-assisted sperm analysis (CASA) was initiated to reduce subjective bias on the motility assessment and to discriminate a series of motility patterns of the sperm. Conventional microscopic methods for sperm evaluation in combination with CASA have allowed obtaining precise information about the sperm quality in pig (Tretipskul et al., 2010). Budworth et al. (1987) found that CASA helped to predict the fertility, and the motility characteristics of spermatozoa were correlated with the capability of spermatozoa in fertilization (Verstegen et al., 2002). The aim of this study was to evaluate the effect of Equex STM Paste supplementation in semen extenders on goat sperm quality after cryopreservation.

2.3 Materials and methods

All chemicals used in this study were purchased from Sigma St Louis, USA, unless otherwise specified.

2.3.1 Animals and semen collection

Fifty semen samples were obtained from six healthy mature bucks: Saanen (n=1), Anglo-Nubian (n=1), Boer (n=1) and Black Bengal bucks (n=3), aged between 2-7 years old. These bucks were housed in a semi-intensive system under preventive and clinical veterinary care. The bucks were fed with concentrate (12% protein) and *ad libitum* of grasses with free access to mineral blocks and water. Ejaculated spermatozoa were collected from each buck by an artificial vagina once a week. Only semen samples with adequate motility (>60%) and sperm concentration (>1,500 x 10^6 spermatozoa/ml were used.

2.3.2 Semen extenders

The basic semen extender in this experiment was Tris (hydroxymethy laminomethane)- citric acid-fructose (TCF) solution (250 mM Tris, 90 mM citric acid, and 70 mM fructose, all were purchased from BDH, Poole, UK) (Chang et al., 2006). The semen extender was adjusted to pH 7.0-7.2 and 375 mOsm/kg. TCF (free of egg yolk and glycerol) was used as sperm washing solution. To make a freezing medium, 10% (v/v) egg yolk and 14% (v/v) glycerol (Sigma, Steinheim, Germany) with or without 1% (v/v) Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA) were added into the TCF.

2.3.3 Freezing and thawing of spermatozoa

Each semen sample was placed in a centrifuge tube, diluted with warm washing solution (37°C), at 1:9 ratios, and followed by centrifugation at 940 x g for 10 minutes. The supernatant was discarded and the semen pellet was dispersed in the leftover medium and divided into two aliquots. They were diluted with glycerolated 10% (v/v) egg yolk Tris-citric-fructose extender with (E+) or without (E-) 1% Equex STM Paste, and the concentration was adjusted to 200 x 10^6 sperm/straw (800 x 10^6 sperm/ml). The diluted semen was equilibrated at 4°C for 4 h. After equilibration, the samples were loaded into 0.25 ml French mini straws (Minitüb, Landshut, Germany). The filled straws were placed horizontallyapproximately 4 cm above the liquid nitrogen level for 10 min in a styrofoam box, before being plunged into the liquid nitrogen. Thawing process of frozen semen was done in a 37°C water bath for 30 seconds (Deka and Rao, 1987). The

post-thaw semen samples were assessed for motility, morphology, viability, acrosome integrity and membrane integrity, before processing and after freezing and thawing. Using CASA, all post-thaw samples were evaluated for motility characteristics after 0 (5 min post-thawing), 1, 2 and 3 h incubations (37°C).

2.3.4 Semen evaluation

2.3.4.1 Assessment of volume, pH and motility: The semen was evaluated immediately after collection by means of volume (graduated tube), pH (pH-Indicator paper, Neutralit®, Merck, Germany), mass movement and progressive motility. The motility of fresh semen was evaluated under a phase-contrast microscope at 100x magnification (microscope model, town, Japan).

2.3.4.2 Assessment of sperm viability: The viability of the spermatozoa was evaluated from a sperm smear stained with eosin–aniline blue. The examination was performed using a bright-field illumination microscope at 1000x magnification (Peterson et al., 2007).

2.3.4.3 Assessment of sperm concentration and sperm morphology: The sperm concentration was determined by a hemocytometer (Neuber, town Germany) after 1:200 dilution. Morphology of 500 sperm heads was evaluated after William's staining and then examined under a light microscope at 1000x magnification (Williams, 1920). A total of 200 spermatozoa fixed in formal saline solution were examined for tail morphology under a phase-contrast microscope at 400x magnification.

2.3.4.4 Assessment of acrosomal integrity: A fluorescein isothiocyanated peanut agglutinin (FITC-PNA) staining was use to evaluate the acrosome integrity as previously described by Axnér et al. (2004), with minor modifications. A 2 µl sperm suspension was smeared onto a glass slide, and then the sperm membrane was permeabilized with 95% ethanol for 30 sec. FITC-PNA (100 µg/ml in PBS) was mixed with propidium iodide (PI, final concentration 18 µM), and the mixture was spread over the sperm smear. The slides were incubated in a moist chamber at 4°C for 30 min then rinsed with 4°C distilled water before air drying. The slides were then evaluated using an epifluorescent microscope (BX51; Olympus, Shinjuku, Japan). At least 200 sperm

per sample were evaluated. Spermatozoa with intensively bright fluorescent acrosomal cap were indicated as acrosome intact spermatozoa.

2.3.4.5 Assessment of functional membrane integrity: The functional membrane integrity was evaluated by the hypo-osmotic swelling test (HOST). An aliquot of semen (2 µl) in 198 µl of hypo-osmotic solution (100 mOsm/kg) was incubated 1 h at 37°C, and percentage of spermatozoa population with swollen tail (sHOST) was scored under a phase-contrast microscope (400x magnifications) (Salvador et al., 2006). A total of 200 spermatozoa were evaluated in at least five different fields. Spermatozoa were classified as swelled (coiled) according to description used by Revell and Mrode (1994).

2.3.4.6 Thermal resistance test: The motility of frozen-thawed semen was evaluated by CASA (IVOS model 12.3, Hamilton-Thorne Biosciences, Bevery, MA, USA). The motility was evaluated 4 times post thawing using CASA coupled with a phase-contrast microscope after incubation at 37°C for 0 (5 min post thawing), 1, 2 and 3 h. To perform CASA, the frozen semen sample was thawed and first diluted 1:9 with phosphate buffered saline. A 10 µl drop of the diluted sample was placed onto a pre-warmed 2X-CEL chamber slides (Hamilton-Thorne, Inc., MA, USA), which was placed on the thermostable plate heated to 37°C prior to the analysis.

2.3.4.7 Computer-assisted-sperm analysis (CASA): The frame rate for semen analysis was set up at 45 frames at 60 Hz. The post-thawed sperm were analyzed for total motility (MOT, %), progressive motility (PMOT, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (a ratio of VSL/VAP; STR, %) and linearity (a ratio VCL/VAP; LIN, %). Each semen sample was scanned in three different selected fields, and the mean value of the three fields was used for statistical analysis.

2.3.5 Statistical Analysis: The results are presented as mean±SD. The differences of post-thaw semen quality between the E+ and E- group were analyzed

based on a student t-test using SAS statistical software (SAS version. 9.0 Cary, N.C., USA) at the significance level of $P<0.05$.

2.4 Results

The quality characteristics of fresh goat semen were in a normal range by means of total volume (1.00±0.38 ml), pH (7.22±0.25), sperm concentration (2,803.8±1,006.7x10^6 spermatozoa/ml), mass movement (3.12±0.72) and motility (71.6±6.24%). Over all, cryopreservation significantly reduced sperm quality compared to fresh spermatozoa by means of sperm plasma membrane, % normal tail sperm, acrosomal membrane integrity and also the functional membrane integrity (sHOST positive spermatozoa). Equex STM paste significantly improved post-thaw quality of goat spermatozoa in terms of the sperm viability ($P=0.0002$) and also the percentage of 'normal-tail' spermatozoa ($P<0.0001$) when compared with no-Equex STM Paste freezing extender (Table 1). The number of acrosome-intact spermatozoa in E+ group was higher than that in E- group but the difference was not significant (Table 1). However, the percentage of normal head and sHOST were decreased in E- group with no significant difference (Table 1). To confirm the positive effect of Equex STM Paste supplementation on post-thaw sperm quality, the thermal resistance test evaluated by CASA was performed. The analysis indicated that the percentage of post-thawing motility (MOT and PMOT) in E+ was higher than E- group (Table 2). Considering at 0 h after thawing, MOT in E+ group were significantly higher ($P=0.0009$). At other incubation time, E+ group provided the better results in both MOT and PMOT except at the end point. In addition to sperm motility, freezing and thawing adversely affected the movement patterns of goat semen, irrespective to the presence of Equex STM Paste in the semen freezing extender. The percentage of VAP, VSL, VCL and ALH in both groups gradually decreased, whereas the BCF, STR and LIN remained unchanged over the incubation times. No significant difference in VAP, VSL, VCL, STR and LIN between E+ and E- groups was observed (Table 2). However, BCF in E- group was significantly higher than E+ group when goat semen was incubated at 0 ($P=0.0012$) and 2 h ($P=0.0209$). Moreover, ALH was higher in E+ group at 0, 1 and 3 h (Table 2).

Table 1 The seminal characteristics of fresh and post-thaw semen with (E+) and without (E-) Equex STM Paste (mean percentage±SD).

Semen quality	Fresh	E-	E+
Viability	79.96±10.13 [a]	31.58±21.74 [b]	43.20±14.43 [c]
Normal tail	91.62±5.83 [a]	73.33±9.97 [b]	89.90±5.36 [c]
Normal head	98.85±0.93 [a]	98.43±1.51 [a]	98.41±1.09 [a]
Acrosome intact	86.22±8.64 [a]	65.88±19.63 [b]	74.30±14.93 [b]
sHOST	71.25±14.55 [a]	36.36±14.51 [b]	34.46±12.33 [b]

The different superscripts ([a, b, c]) with in the same row denote values that differ statistically significant ($P<0.05$)

Table 2 The motility characteristics (MOT, PMOT, VAP, VSL, VCL, ALH, BCF, STR and LIN) of semen frozen with freezing extender supplemented with (E+) and without Equex STM Paste (E-). The semen was thawed and incubated for 0 (5 minutes post-thawing), 1, 2 and 3 h.

Parameter	0 h		1 h		2 h		3 h	
	E-	E+	E-	E+	E-	E+	E-	E+
MOT (%)	27.84±23.27[a]	44.12±14.56[b]	19.20±20.40	25.08±10.57	16.40±17.74	16.44±8.79	13.36±16.99	11.44±8.79
PMOT (%)	7.32±5.79	10.04±4.32	5.12±5.54	6.56±4.20	4.24±5.12	4.84±3.60	3.28±4.64	3.04±3.31
VAP (μm/s)	82.57±20.37	82.49±12.19	75.68±19.23	72.94±12.48	72.04±16.84	65.03±13.06	64.49±18.57	57.90±14.36
VSL (μm/s)	57.23±10.81	53.83±9.33	51.74±12.51	49.79±11.61	49.08±13.83	45.27±11.95	44.81±15.57	38.97±11.38
VCL (μm/s)	170.86±28.29	164.01±24.04	149.22±36.87	141.94±23.54	142.26±21.01	127.55±20.57	128.70±30.73	119.11±23.81
ALH (μm)	7.56±0.95	7.59±0.58	7.08±1.92	7.33±0.87	6.73±1.67	6.69±0.83	6.30±1.08	6.60±1.35
BCF (Hz)	32.92±2.85[a]	30.79±2.49[b]	33.11±4.66	32.17±2.78	34.24±3.64[a]	31.99±4.14[b]	34.50±5.36	31.32±5.47
STR (%)	64.08±4.84	62.96±4.86	65.96±7.66	64.88±7.93	65.72±8.03	65.12±6.78	65.00±8.63	63.96±8.46
LIN (%)	35.64±4.87	33.80±4.35	37.44±8.07	35.76±5.72	36.88±7.35	35.40±4.51	35.96±8.10	33.88±6.04

Data presented as mean ± standard deviation. The Different superscripts ([a, b]) denote values that differ statistically significant ($P<0.05$)

2.5 Discussion

In this study, supplementation of Equex STM Paste in semen freezing extender improved the quality of frozen-thawed goat sperm, although the frozen-thawed semen quality in E+ group was still significantly lower than non-frozen sperm (fresh semen). It has become clear that suboptimal freezing induces irreversible cellular damages in particular at the levels of sperm plasma membrane (Ortman and Rodriguez-Martinez, 1994), acrosomal membrane (Jones and Martin, 1973) and sperm mitochondria (Watson, 1995), etc. As a result, pregnancy rates after transcervical insemination in goat remain poor compared to the direct deposition of frozen semen to the uterine horn via a laparoscopic insemination (Sohnrey and Holtz, 2005). For this reason, improving techniques aimed specifically at cryopreserving goat sperm without significant loss of sperm quality are sought to be important for goat breeding industry. Our results indicated that Equex STM Paste protected the sperm against cryoinjury in terms of MOT, PMOT, sperm viability, acrosome integrity and tail morphology, which are in an agreement with previous studies in other species including pig (Pursel et al., 1978; Buranaamnuay et al., 2009), dog (Rota et al., 1997; Peña and Linde-Forsberg, 2000), cat (Axnér et al, 2004) and horse (Martin et al., 1979; Jimenez, 1987). For goat semen, beneficial effects of trehalose-egg yolk extender containing sodium dodecyl (lauryl) sulphate (SDS) were reported on acrosome integrity and motility (Aboagla and Terada, 2004[a,b]). Equex STM Paste gives similar result to SDS by improving not only acrosome integrity and motility but also viability and normal tail spermatozoa. Equex STM Paste acts as a detergent which has SDS as an active compound. It improves the post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell to protect spermatozoa against the toxic effects of glycerol during the freeze-thaw process (Martin et al., 1979; Arriola and Foote, 1987). In the present study, the percentage of intact acrosome spermatozoa was higher in E+ than E- group. The cryoprotective effect of Equex STM Paste on the acrosomal membrane is crucial since the presence of intact acrosome is necessary during the fertilization process and has been reported to highly correlate with fertility of frozen-thawed semen (Saacke and White, 1972). However, the beneficial

effect of Equex STM Paste on intact acrosome was only observed when the egg yolk was also present. The Equex STM Paste is therefore believed to act via the modifications of egg yolk lipoproteins (Peña and Linde-Forsberg, 2000; Arriola and Foote, 1987). The percentage of post-thawed spermatozoa with normal membrane integrity was decreased in the extender supplemented with Equex STM Paste. Although frozen-thawed sperm in E+ group demonstrated a higher viability and motility (Table 1 and 2), the motility of these frozen-thawed spermatozoa gradually decreased over the incubation times. It seems likely that prolonged incubation of sperm in freezing extender supplemented with Equex STM Paste has a negative effect on sperm membrane/viability probably by the toxic effect of Equex STM Paste (Axnér et al., 2004). Given that the freezing extender is diluted with uterine fluid following artificial insemination, the Equex STM Paste/SDS supplementation will therefore not render the cytotoxicity in the uterus (Axnér et al., 2004). In this study, we used CASA for evaluation of sperm motility since it has been demonstrated to be a precise and objective method in assessing motility patterns (Budworth et al., 1987; Donnelly et al., 1998; Dorado et al., 2009). For example, PMOT has becomean important indicator for the sperm fertilizability because this parameter positively correlates to the pregnancy rates (Donnelly et al., 1998). Equex STM Paste enhanced the ability of spermatozoa to sustain in the incubation at temperatures close to the female body temperature for 2 h. This is similar to a report in cat which Equex STM Paste decreased the longevity of sperm during thermal resistant test (Axnér et al., 2004). However, this result is contradictory to other species such as dogs (Rota et al., 1997), cattles (Arriola and Foote, 1987), and sheep (Akourki et al., 2004). Interestingly, it was found that an addition of Equex STM Paste did not alter the sperm motility pattern (VAP, VAC, VSL, STR and LIN) between E- and E+ groups. These motility patterns correlated with fertilizing ability of spermatozoa (Verstegen et al., 2002). However, sperm frozen with Equex STM Paste had better sperm head movement pattern which is also believe to correlate to the pregnancy outcome. BCF and ALH were significantly higher than E- group. BCF was significantly higher than E- group when goat sperm were incubated at 0 (P=0.0012) and 2 h (P=0.0209), while ALH was higher in E+

group (0, 1 and 3 h). Although these motility patterns have been reported to correlate to the characteristics of sperm during fertilization (Aitken et al., 1985; Jeulin et al., 1996; Sukcharoen et al., 1996; Donnelly et al., 1998), it is still not possible to make any further conclusions that these E+ frozen-thawed sperm still retain their fertilizability in vivo until pregnancy rate after artificial insemination is examined.

2.6 Conclusion

In conclusion, the beneficial effects of Equex STM Paste for MOT, PMOT, viability, the percentage of normal tail, and intact acrosome were found especially during the first 2 h after thawing. However, the long incubation of goat sperm in Equex STM paste has an adverse effect during the 3 h thermal resistant test. The optimal concentration for frozen goat semen and the fertilizability of sperm frozen with Equex STM Paste *in vivo* remains to be clarified.

CHAPTER III

GLYCEROL CONCENTRATION AFFECTS ON QUALITY AND LONGEVITY OF POST-THAW GOAT SEMEN

3.1 Abstract

This study determined the effects of glycerol concentration (7, 10 and 14% (v/v)) supplemented into the freezing medium on post-thaw sperm qualities. Semen collected from 3 Black Bengal bucks and then pooled to reduce individual variation. The motility, viability, morphology and acrosome integrity were evaluated before and after cryopreservation. Computer-assisted sperm analysis was used to assess the sperm motion patterns at 0, 1, 2 and 3 h post-thawing. The laparoscopic artificial insemination was performed to test the fertilizing ability of frozen-thawed semen. The spermatozoa cryopreserved with 10% glycerol tended to have a higher percentage of motility, progressive motility, spermatozoa with intact acrosome membrane and live spermatozoa especially when examined at 1 h after thawing. Motion characteristics in all groups were similar except that the beat cross-frequency of spermatozoa frozen with 14% glycerol was significant lower than 10% glycerol group (P=0.0211). The laparoscopic AI using frozen-thawed semen obtained from 10% glycerol group yielded 60% (3/5) conception rate and three live offspring. In conclusion, glycerol concentration affects on qualities and longevity of post-thaw goat semen. The freezing medium containing 10% glycerol sufficiently protected goat spermatozoa against cryoinjury during freezing and thawing, and provided an acceptable pregnancy rate.

3.2 Introduction

The application of artificial insemination (AI) is important for genetic improvement programs in goat. However, pregnancy rate following AI with frozen-thawed semen is generally poorer than that obtained from fresh semen (Sohnrey and Holtz, 2005). This indicates that freezing and thawing inevitably induces cryodamage to spermatozoa, thus modification of freezing media to improve the goat sperm viability is required. To

date, various freezing media and protocols have been developed aimed specifically at reducing cryoinjury and, in turn, improving the quality of post-thawed spermatozoa. Among factors affecting the freezing ability of spermatozoa, supplementation of freezing medium with various types of cryoprotectant plays a central role in minimizing the physical and chemical stresses occurred during cryopreservation procedure (Purdy, 2006; Peterson et al., 2007). Although it is generally accepted that high concentration of cryoprotectant reduces lethal intracellular ice formation (Mazur and Kleinhans, 2008), this is frequently consequent with probable toxicity of the particular cryoprotectant (Fahy, 2010). Furthermore, other chemical components added into the freezing medium, in particular non-penetrating cryoprotectant such as lactose (Singh et al., 1995; Chang et al., 2006), trehalose (Aboagla and Terada, 2003; Peterson et al., 2007), and surfactants either sodium dodecyl sulfate (SDS) (Aboagla and Terada, 2004[a, b]) or Equex STM paste (Anakkul et al., 2011) have also been reported to affect on the freezing ability of goat semen.

Glycerol has three hydroxyl (OH) groups which their hydrogen atoms are likely to form H-bonding with the oxygen atoms of the phosphate groups of membrane phospholipids (Kundu et al., 2000). It acts as a membrane-permeable cryoprotectant, resulting in dehydration of spermatozoa due principally to the osmotically driven efflux of intracellular water (Watson, 1995; Amann, 1999). This prevents the sperm from supercooling and also decreases intracellular ice formation. It is the most widely used as a membrane-permeable cryoprotectant for preserving mammalian spermatozoa (Tuli and Holtz, 1994). This cryoprotectant is superior for protecting buck spermatozoa during cryopreservation to other chemicals (Leboeuf et al., 2000; Purdy, 2006; Peterson et al., 2007). However, suitable amount of glycerol added into the freezing medium remains controversial.

High concentrations (10-14%, v/v) were applied in an early study of buck semen cryopreservation (Ritar et al., 1990[b]) but, at the present time, the glycerol was reduced to 3-9% (v/v) (Leboeuf et al., 2000). Moreover, the amounts of glycerol added into the freezing extender are likely to differ between the breeds and individual males (Holt,

2000[b]), for examples, in pigs (Waterhouse et al., 2006), marsupials (Taggart et al., 1996) and African antelopes (Loskutoff et al., 1996). The excess amount of glycerol induces osmotic damage to spermatozoa, hence reducing post-thaw semen quality (Holt, 2000[a]). On the other hand, too low glycerol concentration poorly protects the spermatozoa against cryoinjury (Silva et al., 2002). Therefore, appropriate glycerol concentration in the freezing medium, as well as a long-term consequence such as frozen-thawed semen longevity and conception rate following AI should be cautiously determined. In this study, we used semen from Black Bengal bucks as a model since they have high climate-adaptability, fertility and superior skin quality (Husain et al. 1996). However, spermatozoa from Black Bengal buck are quite sensitive to cold stress, and large variability of post-thaw sperm motility (5 to 50%) have been reported (Biswas et al., 2002; Afroz et al., 2008).

This study was carried out to investigate the effects of different glycerol concentrations on the quality and longevity of frozen-thawed Black Bengal buck spermatozoa. The pregnancy rate after laparoscopic artificial insemination with frozen-thawed semen was additionally examined.

3.3 Materials and methods

3.3.1 Chemical reagents

All chemicals used in this study were purchased from Sigma St Louis, USA, unless otherwise specified.

3.3.2 Experimental animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC), Chulalongkorn University (Approval No. 11310030). Three Black Bengal bucks, aged between 2 to 4 years old, were used in this experiment. The animal facility is located at the Veterinary Student Training Center, Nakhon Pathom Province (latitude 13°N and longitude 100°E). The animals were fed with concentrates containing 14% (w/w) protein, *ad libitum* of grass with free access to mineral salt blocks and water.

3.3.3 Semen processing and cryopreservation

Semen from three bucks was collected twice a week using an artificial vagina during May to August 2011 (summer to rainy season). The ejaculate volume was evaluated from a graduated collection tube. Semen pH was evaluated by a pH-indicator paper (Neutralit®, Merck, Darmstadt, Germany). Mass movement and progressive motility of fresh and equilibrated semen were subjectively evaluated under a phase contrast microscope (CX41RF; Olympus, Tokyo, Japan). Sperm concentration was calculated using a hemocytometer (Neubauer, Boeco, Germany) after a 1:400 dilution with formal saline solution. The ejaculates with an acceptable progressive motility (>65%) and concentration (>1,500 x 10^6 spermatozoa/ml) were selected and pooled for further processes.

Tris-citric acid-fructose (TCF) solution (pH 7.0 to 7.2), consisted of 250 mM Tris (hydroxymethyl animomethane), 90 mM citric acid, 70 mM fructose (BDH, Poole, UK), 100 IU/ml penicillin G and 100 µg/ml streptomycin, was supplemented with 10% (v/v) fresh hen egg yolk and 1% (v/v) Equex STM paste (Nova Chemical Sales Inc., Scituate, USA). The extender containing different glycerol concentrations (7, 10 and 14% glycerol (v/v)) was then finally prepared. To prepare each 10 ml of three different glycerol concentrations in extender in brief, the TCF solution (7.5x3 ml) was diluted with egg yolk (1x3 ml) and Equex STM Paste (0.1x3 ml). After mixing well, the medium was separated into 3 aliquots (7.5 ml each). The three amounts of glycerol (0.7, 1 and 1.4 ml) and TCF (0.7, 0.4 and 0 ml) were then added to each aliquot, respectively.

Pooled samples were diluted with TCF (1:9 at 37°C) and then centrifuged at 940 x g for 10 min at room temperature in order to remove the seminal plasma. The supernatant was discarded, and the sediment containing spermatozoa was divided into three equal aliquots, each was diluted with extender containing 7, 10 or 14% glycerol. The sperm concentration was finally adjusted to 400 x 10^6 spermatozoa/ml. The diluted semen was then equilibrated at 4°C for 4 h. After equilibration, the semen was loaded into 0.25 ml French mini straws (Minitüb, Landshut, Germany), and the open-end was sealed with polyvinyl powder. The straws were placed horizontally in liquid nitrogen

vapor at 4 cm above the liquid nitrogen level in a styrofoam box for 10 min and then plunged directly into liquid nitrogen. Semen thawing was performed at 37°C in a water bath for 30 sec (Deka and Rao, 1987; Leboeuf et al., 2000).

3.3.4 Assessment of semen quality

The assessment of semen quality was performed as described in chapter II

3.3.5 Laparoscopic artificial insemination and pregnancy diagnosis

To examine the fertilizing ability of frozen-thawed semen provided the best freezing protocol from the experiment, which was 10% glycerol, five female 75% Saanen does, aged between 2-3 years old, were used for laparoscopic artificial insemination.. The estrus was synchronized by an intravaginal progesterone sponge containing 65 mg of Medroxyprogesterone (Sincro-gest sponges®; Laboratorios Ovejero, Leon, Spain) for 13 days. An injection of 300 IU pregnant mare serum gonadotropin (Sincro-gest PMSG®; Laboratorios Ovejero, Leon, Spain) was done two days before the sponge removal. The estrus detection was carried out using a teaser buck and the insemination was done at 24 h (Martinez-Rojero et al., 2007) after the standing estrus. The does were sedated with xylazine hydrochloride (0.1 mg/kg) and ketamine (4 mg/kg), and were inseminated using laparoscopic technique as described by Mobini et al. (2002) with some modification. In brief, the does were sedated and placed in dorsal recumbency, the animal's head downwards. The carbon dioxide (CO_2) was inflated in the abdomen via verres needle. Two incisions were made at approximately 5 cm cranial to the udder and 4 cm to either side of the midline to facilitate the penetration of trocar and cannula. The 5 mm laparoscope (Schölly, Denzlingen, Germany) was inserted through the first incision, and then post-thaw spermatozoa were inseminated through the other incision. A total of $120x10^6$ frozen-thawed spermatozoa were deposited into the lumen of both uterine horns ($60x10^6$ spermatozoa per horn). Pregnancy was diagnosed at 45 days post insemination by a real-time B mode ultrasound (HS-2000, Honda Electronics CO., LTD., Aichi, Japan). The numbers of live birth were also recorded

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3.3.6 Statistical analysis

The results were presented as means with pooled SE. Semen characteristics of pooled samples were descriptively analyzed. The comparisons among semen parameters were analyzed by general linear model procedure (GLM) using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). The values with $P<0.05$ were considered statistically significant.

3.4 Results

The semen characteristic data of pooled samples were summarized in Table 3. The mean volume per ejaculate of fresh semen from all bucks was 0.59±0.5 ml. Following the addition of freezing extender, the spermatozoa were slowly cooled at 4°C for 4 h prior to spermatozoa evaluation. It was found that different concentrations of glycerol supplemented in the freezing extender did not affect the spermatozoa quality, in terms of viability, acrosome integrity, percentage of normal morphology (Table 4, $P>0.05$). There were also no significant differences among glycerol concentrations for post-thaw viability and percentages of normal sperm morphology in all incubation times (Table 4). Nevertheless, spermatozoa cryopreserved with 10% glycerol tended to have a higher percentage of live spermatozoa when compared to other concentrations. This finding coincided with the results that the 10% glycerol preserved integrity of acrosomal membrane better than other concentrations especially when examined at T1. Although MOT and PMOT gradually decreased along the incubation time, buck spermatozoa frozen with 10% glycerol showed the superior results of MOT and PMOT compared to the 7% and 14% glycerol groups in all evaluation times (Table 5). The significant differences were shown at T0 for PMOT and at T0 and T1 for MOT ($P<0.05$). Motion characteristics in all glycerol groups were similar except that the beat cross frequency of spermatozoa frozen with 14% glycerol was significant lower than 10% glycerol group ($P=0.0211$, Table 5). The spermatozoa frozen with the 10% glycerol protocol retained the fertilizing ability since the ultrasound scanners diagnosed 60% of pregnancy (three of five does) with 100% accuracy. This resulted in a total of three live offspring.

Table 3 Semen characteristics of pooled samples obtained from Black Bengal goat bucks

Semen parameter	Mean±SE
pH	7.6±0.5
Mass movement (0-4 score)	2.6±0.9
Motility (%)	68.1±1.8
Concentration (x 10^9/ml)	3.6±1.3
Viability (%)	77.3±2.4
Normal head morphology (%)	98.5±1.2
Normal tail morphology (%)	90.4±2.3
Acrosome intact spermatozoa (%)	88.2±2.3

Table 4 The mean percentages of viability, acrosome intact, normal head and normal tail morphology of equilibrated and post-thaw spermatozoa frozen with different glycerol concentrations (7, 10 and 14%)

Parameter	Viability (%)	Intact acrosome (%)	Normal head morphology (%)	Normal tail morphology (%)
Post-equilibration				
7% glycerol	65.4	80.5	98.9	86.9
10% glycerol	66.5	79.4	99.3	85.1
14% glycerol	61.09	80.4	99.4	84.1
Post-thawing (T0)				
7% glycerol	33.3	83.7	99.2	91.4
10% glycerol	40.4	87.8	99.8	90.3
14% glycerol	31.4	86.8	98.9	91.1
Post-thawing (T1)				
7% glycerol	14.1	74.7^{ab}	99.2	91.6
10% glycerol	21.7	80.1^{a}	98.8	91.9
14% glycerol	21.5	70.7^{b}	99.2	91.4
Post-thawing (T2)				
7% glycerol	12.3	66.9	99.1	91.4
10% glycerol	14.8	69.5	98.9	92.0
14% glycerol	14.3	67.2	99.0	93.2
Post-thawing (T3)				
7% glycerol	9.7	64.7	99.3	91.9
10% glycerol	10.4	66.6	98.7	91.9
14% glycerol	10.3	57.3	99.0	93.2
SE	1.44	1.40	1.07	0.48

The statistical analysis was used to compare the values obtained from different glycerol concentrations. Within the same sperm parameter, the different superscripts denote values that differ statistically significant ($P<0.05$). SE indicates standard error.

Table 5 The motility characteristics of post-thaw spermatozoa frozen in freezing extender supplemented with 7, 10 and 14% glycerol

Parameter	MOT (%)	PMOT (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hertz)	STR (%)	LIN (%)
Post-thawing (T0)									
7% glycerol	30.9[a]	7.6[a]	84.1	56.1	161.7	7.5	32.7	64.6	35.7
10% glycerol	42.3[b]	12.0[b]	90.8	62.4	173.7	7.4	32.9	66.0	36.0
14% glycerol	37.4[ab]	8.3[a]	79.5	51.1	157.7	7.2	30.6	64.4	35.9
Post-thawing (T1)									
7% glycerol	18.7[a]	6.2	70.3	53.0	133.0	6.9	32.4	72.9	41.9
10% glycerol	28.3[b]	9.7	77.5	55.9	149.4	7.3	32.1	69.3	38.0
14% glycerol	18.9[a]	6.2	69.2	50.1	133.5	6.9	32.7	69.8	38.6
Post-thawing (T2)									
7% glycerol	11.4	4.0	64.7	48.5	120.2	6.2	31.2	71.8	40.1
10% glycerol	17.7	6.2	62.9	46.4	122.6	5.9	34.4	68.8	37.7
14% glycerol	14.3	4.8	58.8	43.3	113.3	6.3	32.7	69.6	38.1
Post-thawing (T3)									
7% glycerol	7.1	2.1	51.8	38.7	102.4	5.6	31.0[ab]	72.7	41.3
10% glycerol	11.6	4.0	57.1	41.7	103.2	5.7	32.3[a]	68.8	38.6
14% glycerol	10.0	3.3	52.3	38.5	102.6	5.8	28.4[b]	71.5	41.7
SE	1.29	0.66	2.10	1.83	3.80	0.24	0.67	1.22	1.28

MOT, motility; PMOT, progressive motility; VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of the lateral head displacement; BCF, beat cross-frequency; STR, straightness; LIN, linearity. Within a column and time point, different superscripts denote values that differ statistically significant ($P<0.05$). SE indicates standard error.

3.5 Discussion

Goat sperm are sensitive to cryopreservation as sperm quality and pregnancy are usually poorer than that obtained from non-cryopreserved sperm (Medeiros et al., 2002; Apu et al., 2012). In this study, we found that the amount of glycerol supplemented into the freezing extender affected the post-thaw sperm quality. Following semen collection, some semen parameters were slightly different compared with previous reports such as lower in motility but higher in semen volume and concentration (Biswas et al., 2002; Apu et al., 2008). This may be a consequence from the variation of age, semen collection method, living condition and individual effect (Apu et al., 2008). Overall, the observed semen characteristics in this study were typical of sexually mature Black Bengal bucks as previously reported (Khan, 1999; Apu et al., 2012). This present study indicated that the concentration of glycerol in freezing extender affected the quality and longevity of cryopreserved semen. Addition of 10% glycerol in the TCF extender supplemented with 10% hen egg yolk and 1% Equex STM Paste significantly improves the percentage of PMOT and MOT of spermatozoa after thawing (PMOT and MOT) or until 1 h of incubation (MOT), whereas the lowest sperm quality was observed in extender containing 7% glycerol. The appropriate amount of glycerol increases the membrane fluidity by rearranging the membrane lipid and protein, thus increasing the survival ability of spermatozoa during cryopreservation (Holt, 2000[a]). The range of 3 to 14% glycerol are generally used in freezing extender for several goat breeds (Leboeuf et al., 2000) while 5 and 7% glycerol are frequently reported for Black Bengal breed (Biswas et al., 2002; Afroz et al., 2008; Apu et al., 2012), but we found that the 7% glycerol protocol poorly protected the spermatozoa against cryoinjury during freezing and thawing procedure. This finding was in agreement to other reports indicating that addition of 7% glycerol was not suitable for proper dehydration of the goat sperm cells (Afroz, 2008). By contrast, Biswas et al. (2002) reported that 7% glycerol protocol produced higher post-thaw Black Bengal sperm motility than the 10% glycerol supplementation (51±1% and 7±1%, respectively). The different results may due to the

different freezing extender compositions, for example a difference amount of egg yolk, a difference kind of sugar and an absence of Equex STM Paste.

And also the fact that the latter study used subjective sperm evaluation which variations of 30 to 60% have been reported, while this study used CASA, an objective method (Verstegent et al., 2002) which provides more accurate estimates and has demonstrated to be a useful tool to evaluate kinematics properties of individual spermatozoa (Tuli et al., 1992; Mortimer, 1997; Verstegent et al., 2002). On the other hand, poor sperm quality obtained from a high glycerol concentration (14%) probably cause by an extreme osmotic stress that can damage the cell structures and impair the sperm motility (Deka and Rao, 1986; Gil et al., 2003; Sönmez and Demirci, 2004; Sundararaman and Edwin, 2008).

In this study, glycerol concentration did not significantly affect velocity or speed characteristics (VAP, VSL and VCL) as well as STR and LIN in frozen-thawed semen. Only BCF at T3 showed a significant difference between the 10 and 14% glycerol groups (P=0.0211). BCF is a frequency of sperm head crossing the sperm average path in Hertz. It gives an indication of the flagella beat frequency and assesses the time that flagella beat changes its pattern, which a low of BCF values may impair the penetrating ability of spermatozoa (Mortimer, 1997). We found that BCF values were different, despite of the similar head and tail morphology between groups. It is possible that optimal glycerol and Equex STM Paste concentration used in the freezing extender synergistically protected the spermatozoa plasma membrane (Peña et al., 2003). Although the numbers of inseminated does would have been increased, the pregnancy and kidding rate indicated conclusively that the spermatozoa frozen with the 10% glycerol protocol retained the fertilizing ability. The pregnancy result obtained was in a normal range (20% to 90%) reported in laparoscopic insemination (Mobini et al., 2002).

In conclusion, the glycerol concentration in semen freezing extender affected the quality and longevity of frozen-thawed Black Bengal buck semen. The freezing medium containing 10% glycerol sufficiently protected goat spermatozoa against cryoinjury during freezing and thawing, and provided an acceptable pregnancy rate.

Further examination on other factors that interact and influence on the spermatozoa quality following freezing and thawing remain to be studied.

CHAPTER IV

EQUEX STM PASTE IMPROVES THE FREEZING ABILITY OF GOAT SPERMATOZOA

4.1 Abstract

Freezing and thawing inevitably decrease spermatozoa viability and functions, and the conception following artificial insemination with frozen-thawed semen is usually poor. The aim of this study was to investigate the effects of different concentrations of Equex STM Paste on the quality of goat semen after freezing and thawing. Fresh semen (n=16) was collected from eight bucks every two weeks for 8 consecutive months. Semen samples were pooled, separated into six portions, and then diluted in freezing extender without or with different concentrations of Equex STM Paste (0.25, 0.5, 0.75, 1.0 and 1.25%, v/v). After freezing-thawing, the motility characteristics were assessed by computer-assisted-sperm analysis at 0, 1, 2 and 3 h. All post-thaw samples were also evaluated for viability, morphology, acrosome integrity, DNA integrity and functional membrane integrity. Sperm quality was compared between fresh and frozen–thawed samples as well as between groups after thawing for 5 min (0 h), 1, 2 and 3 h. Supplementation of Equex STM Paste into freezing extender significantly improved spermatozoa quality when compared with the control ($P<0.05$). The 0.5% Equex STM Paste group provided higher motility, viability and % Host-positive spermatozoa than other Equex STM Paste concentrations in all incubation times.

4.2 Introduction

The freezing and thawing process inevitably results in cellular damage, and thus post-thaw semen quality is usually poor when compared with fresh semen. During the past decade, attempts have been made to improve the quality of frozen-thawed spermatozoa by means of modifying freezing medium. Glycerol and egg yolk are most frequently used as cryoprotective agents in goat semen freezing (Ritar and Salamon, 1982; Tuli and Holtz, 1994). However, freezing medium containing egg yolk renders detrimental effects on sperm viability due principally to harmful interactions of egg yolk

and egg yolk-coagulating enzymes present in the seminal plasma (Roy, 1957; Iritani and Nishikawa, 1963; Leboeuf et al., 2000; Purdy, 2006).

Removal of seminal plasma by washing the semen via centrifugation prior to cryopreservation and reducing the amount of egg yolk added to the freezing extender are beneficial (Singh et al., 1995; Leboeuf et al., 1998; Dewit et al., 2000). Nevertheless, an increased rate in the number of loss spermatozoa and damaged spermatozoa plasma membrane has been reported after centrifugation (Corteel, 1981; Azerêdo et al., 2001). Several strategies have currently been used to improve membrane fluidity of the spermatozoa such as modifying the sperm plasma membrane with cholesterol-loaded-cyclodextrin (Klein et al., 1995; Zahn et al., 2002; Farshad et al., 2011) and also using some detergents to alter the interactions between lipoproteins in the egg yolk and plasma membrane resulting in a more accessible lipoproteins to the sperm plasma membrane (Amann and Pickett, 1987).

Sodium dodecyl sulphate (SDS) and Equex STM Paste is a water-soluble anionic detergent that has been supplemented as an additive in the freezing extender (Dewit et al., 2000). The positive effects of Equex STM Paste have been shown especially when egg yolk was presented in the freezing extender (Pursel et al., 1978; Dewit et al., 2000; Aboagla and Terada, 2004[a]). Although the exact concentration of SDS (active ingredient of Equex STM Paste) and Equex STM Paste has not been clearly identified (Peña et al., 2003), they have been demonstrated to be beneficial for goat semen cryopreservation. (Aboagla and Terada, 2003; 2004[a,b]; Chang et al., 2006; El-Kon et al., 2010; Bittencourt et al., 2008; Anakkul et al., 2011). Our preliminary study showed that 1% (v/v) Equex STM Paste supplemented into the freezing extender improved post-thaw quality of goat spermatozoa. However, prolonged incubation (37°C) of goat spermatozoa adversely affected the sperm quality (Anakkul et al., 2011). This finding is in agreement with previous reports indicating that the high percentage of SDS decreased motility and acrosome integrity of cryopreserved spermatozoa (Arriola and Foote, 1987; Dewit et al., 2000; Aboagla and Terada, 2004[a]). Furthermore, Equex STM Paste was also found to negatively affect the sperm membranes during equilibration (Anakkul et al., 2011; Peña

and Linde-Forsberg, 2000). Therefore, the optimal concentration of Equex STM Paste for the cryopreservation of goat semen remains to be studied.

This study aimed at investigating the effects of Equex STM Paste concentrations in freezing extender on post-thaw quality of goat spermatozoa, in term of motility characteristics, viability, morphology, acrosome integrity, DNA integrity and plasma membrane integrity.

4.3 Materials and methods

All chemicals used in this study were obtained from Sigma-Aldrich, St. Louis, USA, unless otherwise specified.

4.3.1 Experimental design

The fresh semen from eight bucks was evaluated for semen quality. The semen samples possessing >70% motility and >1,500 x 10^6 spermatozoa/ml were pooled together and diluted in freezing extender supplemented without or with different concentrations of Equex STM Paste (0.25, 0.5, 0.75, 1.0 and 1.25%, v/v). After freeze-thawing, the motility characteristics were assessed at 37°C by CASA for 0, 1, 2 and 3 h of incubation. Post-thaw samples were also evaluated for viability, morphology, acrosome integrity, DNA integrity and membrane integrity. Sperm quality was evaluated in a time-course series and compared fresh and frozen–thawed samples as well as between the Equex STM Paste concentrations after thawing.

4.3.2 Animals and semen collection

The study was approved by the Institutional Animal Care and Use Committee (IACUC) (Approval No. 11310030), Chulalongkorn University. The semen samples were obtained from eight healthy 75% Saanen bucks approximately 2 years of age. They were housed at the CU-Network for Academic Opportunities and Services, Chulalongkorn University, Nan Province, Thailand (18°N latitude and 100°E longitude), in a free-stall housing under preventive and clinical veterinary care. They were provided a daily feed of silage and concentrates (14% protein) twice a day. Grasses, mineral salt and water were available *ad libitum*.

The semen was collected by artificial vagina every two weeks for eight consecutive months (n=16) during October to May (winter to summer). The fresh semen from each buck was evaluated, within 30 min, for volume, pH and sperm concentration using pH-indicator paper (Neutralit®, Merck, Darmstadt, Germany) and a haemocytometer (Neubauer, Boeco, Germany), respectively. The motility was subjectively evaluated under a phase-contrast microscope at 100x magnification (CX41RF; Olympus, Tokyo, Japan). Only semen samples with adequate motility (>70%) and sperm concentration (>1,500 x 10^6 spermatozoa/ml) were pooled and used in this study. Sperm concentration, motility, viability, morphology, plasma membrane integrity, acrosome integrity and DNA integrity were assessed in the pooled samples to minimize individual variation.

4.3.3. Semen extender

The basic semen extender used in this experiment was Tris (hydroxymethyl-aminomethane)-citric acid-fructose (TCF) solution (BDH, Poole, England) containing 250.0 mM Tris, 90.0 mM citric acid and 70.0 mM fructose. The solution was adjusted to pH 7.0-7.2. TCF (free of egg yolk and glycerol) was used as a washing solution. To prepare freezing medium, ten percent (v/v) each of egg yolk and glycerol were added into the TCF. The Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA) was then supplemented (v/v) into this freezing medium at different concentrations depending on the experimental design (0, 0.25, 0.50, 0.75, 1.00 and 1.25%).

4.3.4. Freezing and thawing of goat semen

Pooled semen was diluted with TCF (1:9) at 37°C and washed by centrifugation at 28° C, for 10 min at 940 x g. After the supernatant was discarded, the semen pellet was divided into six aliquots. They were then diluted to a final concentration of 240 x 10^6 spermatozoa/ml with freezing extenders containing different concentrations of Equex STM Paste (0.25, 0.50, 0.75, 1.00 and 1.25%), thereafter referred to as E0.25, E0.5, E0.75, E1.0 and E1.25 group, respectively. The semen frozen without Equex STM Paste (E0) served as the control.

The semen was frozen as described previously in chapter II. Briefly, the diluted semen was equilibrated at 4°C for 4 h and then loaded into 0.25 ml French mini straws

(Minitüb, Landshut, Germany). The straws containing semen were placed horizontally in liquid nitrogen vapor (about 4 cm above the liquid nitrogen level). After 10 min, they were plunged into the liquid nitrogen and stored until evaluation. For each extender, one straw was randomly thawed by placing the straw in a 37°C water bath for 30 sec.

4.3.5 Sperm evaluation

The assessments for viability, morphology, acrosome integrity, DNA integrity and plasma membrane integrity were performed in fresh pooled samples as well as post-thaw spermatozoa as described in chapter II. Motility characteristics were evaluated by CASA only in post-thaw samples.

DNA fragmentation was investigated by Acridine orange (AO) (Tejada et al., 1984). In brief, the spermatozoa were smeared on a glass slide and allowed to air-dry. They were subsequently fixed overnight in freshly prepared methanol: glacial acetic acid (3:1), then air-dried. The slides were stained with AO staining solution (0.25%, w/v) for 5 min at room temperature. After staining, the slides were rinsed several times with distilled water. Five hundred spermatozoa were evaluated under an epifluorescent microscope (BX51; Olympus, Shinjuku, Japan). The spermatozoa with normal DNA (double-stranded) were typified by green fluorescence, whereas those with damaged or single stranded DNA showed orange or red fluorescence using 490 nm excitation.

4.3.6 Statistical analysis

Statistical analyses were carried out by general linear model procedure (GLM) using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). The least-square mean values were obtained from each class of the factors (treatment, time and treatment*time) and were compared by using least significant different test (LSD). The values with $P<0.05$ were considered significant. The results are presented as mean±SD.

4.4 Results

Prior to freezing, the characteristics of pooled semen were within the normal range as shown in Table 6. Freezing and thawing significantly reduced the quality of goat spermatozoa. However, supplementing Equex STM Paste into the freezing extender significantly improved the sperm quality when compared with the control ($P<0.05$) (Fig. 2 and Table 7). All the Equex STM Paste-treated groups had significantly better post-thaw sperm viability at T0 and T1 than the control. This study also found that the semen frozen with E0.5 yielded the best MOT and PMOT (Figs. 2a and 2b), while higher Equex STM Paste concentrations appeared to decrease motility. The positive results of Equex STM Paste on spermatozoa quality were also in an agreement to the findings that the tail morphology and acrosomal membrane were best preserved when the spermatozoa were frozen in a presence of Equex STM Paste, irrespective the Equex STM Paste concentrations (Table 7). However, there were no significant change in the percentage of spermatozoa with normal head morphology and DNA integrity in all of the groups (Table 7). Equex STM Paste containing extender (E0.25 and E0.50) also positively affected the motility patterns including VAP, VSL and VCL compared with the control and E1.0 and E1.25 extenders (Table 8 and 9). There was no significant difference in BCF among the groups. In general, Equex STM Paste positively affected the longevity of goat spermatozoa, but the protective effects of Equex STM Paste were in a dose dependent manner. E0.5 is the best concentration.

Table 6 Characteristics of pooled semen prior to cryopreservation

Parameter	Mean ± SD
Concentration ($x10^9$ /ml)	3.1 ± 1.4
pH	6.9 ± 0.2
Motility (%)	73.4 ± 2.4
Viability (%)	71.9 ±12.7
Sperm with intact acrosome membrane (%)	91.9 ± 4.9
Sperm with intact plasma membrane (%)	55.3 ± 24.4
Normal head morphology (%)	98.9 ± 0.9
Normal tail morphology (%)	92.5 ± 5.6
Sperm with normal DNA (%)	99.4 ± 1.3

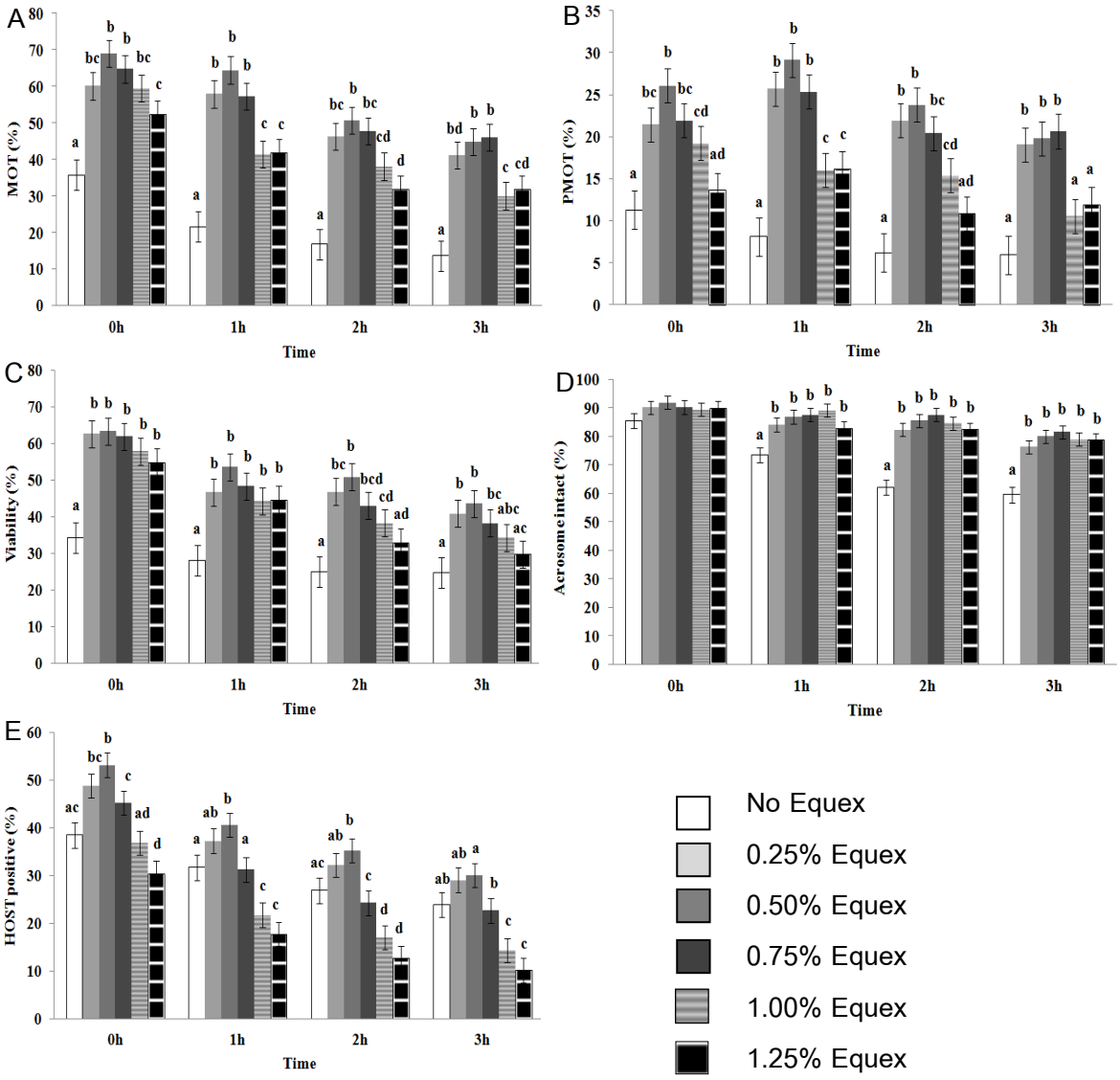

Figure 2 The motility (MOT, A), progressive motility (PMOT, B), viability (C), spermatozoa with acrosome intact (D) and HOST positive spermatozoa (E) of goat semen cryopreserved with 0.25, 0.5, 0.75, 1.0 and 1.25% Equex STM Paste at 5 min (0 h), 1, 2 and 3 h after thawing. Within the same evaluated time, the different superscripts denote values that differ statistically significant ($P<0.05$).

Table 7 The sperm quality of goat semen cryopreserved with 0.25, 0.5, 0.75, 1.0 and 1.25% Equex STM Paste at T0 compared with fresh semen

Parameter	Fresh	Concentration of Equex STM Paste (%)					
		0.00	0.25	0.50	0.75	1.00	1.25
MOT	73.2±2.5[a]	35.7±12.0[b]	60.1±11.5[b]	68.9±10.4[a]	64.7±14.8[b]	59.5±14.7[b]	52.3±16.4[b]
Viability	73.3±9.1[a]	34.3±15.9[b]	62.7±13.2[b]	63.4±11.9[b]	61.9±8.3[b]	57.9±10.7[b]	54.9±13.2[b]
Normal head	98.8±0.9	99.5±0.5	99.4±0.5	99.1±1.0	99.4±0.7	99.4±0.5	99.4±0.7
Normal tail	92.1±6.0[a]	78.6±4.7[b]	89.2±2.9[a]	90.0±3.0[a]	89.1±6.6[a]	90.8±1.8[a]	89.5±2.7[a]
Acrosome intact	91.6±5.0[a]	85.6±6.1[b]	90.1±4.4[a]	91.8±3.6[a]	90.2±4.6[a]	89.4±5.8[a]	90.0±5.7[a]
HOST	45.8±23.6[a]	38.5±12.3[a]	48.8±13.4[a]	53.1±10.5[a]	45.2±9.6[a]	36.8±11.1[a]	30.5±10.7[b]
Normal DNA	99.4± 1.4	99.8±0.2	99.8±0.3	99.7±0.3	99.8±0.2	99.9±0.1	99.9±0.2

Within row, the different superscripts ([a, b]) denote values that differ statistically significant ($P<0.05$).

Table 8 The motility characteristics (VAP, VSL and VCL) of goat semen cryopreserved with 0.25, 0.5, 0.75, 1.0 and 1.25% Equex at 5 min (T0), 1 (T1), 2 (T2) and 3 h (T3) after thawing

Parameter	Time	Concentration of Equex STM Paste					
		0%	0.25%	0.50%	0.75%	1.00%	1.25%
VAP	0	99.0±14.7^{ac}	112.7±7.8^{b}	111.8±7.8^{b}	107.7±7.7^{ab}	100.1±8.9^{ac}	97.4±6.6^{c}
(μm/s)	1	98.6±16.7^{ab}	109.6±8.8^{a}	108.7±11.2^{a}	97.4±25.1^{b}	95.6±9.0^{b}	89.3±11.6^{b}
	2	85.9±18.8^{ac}	102.3±9.0^{b}	103.1±13.1^{b}	97.4±9.0^{bc}	91.0±12.1^{c}	83.7±9.6^{a}
	3	87.1±23.1^{ac}	98.7±11.7^{b}	92.1±13.9^{ab}	90.5±9.8^{ab}	77.1±16.9^{c}	77.9±12.1^{c}
VSL	0	66.6±13.5^{ac}	79.4±8.5^{b}	79.8±9.0^{b}	74.9±8.3^{ab}	69.0±9.5^{ac}	62.7±9.4^{c}
(μm/s)	1	72.3±15.6^{ac}	84.5±12.2^{b}	83.1±13.6^{b}	79.9±7.1^{ab}	70.6±10.9^{c}	66.9±9.3^{c}
	2	59.6±22.1^{a}	79.6±9.7^{b}	80.4±12.0^{b}	73.8±10.0^{bc}	67.9±11.9^{c}	60.4±9.4^{a}
	3	66.2±18.4^{ac}	76.7±10.3^{b}	70.7±12.3^{ab}	70.0±11.4^{ab}	56.8±13.7^{c}	57.9±11.9^{c}
VCL	0	188.4±25.6^{a}	218.8±18.7^{b}	216.4±16.9^{b}	210.1±19.5^{b}	193.8±22.6^{a}	190.8±16.9^{a}
(μm/s)	1	182.9±31.7^{a}	194.3±12.1^{a}	192.4±21.3^{a}	188.3±9.8^{a}	179.4±15.3^{ab}	166.7±23.4^{b}
	2	159.1±32.2^{ac}	178.4±18.1^{b}	180.3±22.7^{b}	175.3±16.7^{ab}	166.2±21.3^{abc}	157.6±13.6^{c}
	3	159.8±36.4^{ab}	170.8±23.4^{a}	164.1±22.1^{a}	159.6±16.4^{ab}	143.6±25.7^{b}	145.3±19.5^{b}

Within the same parameter, the different superscripts denote values that differ statistically significant ($P<0.05$)

Table 9 The motility characteristics (ALH and BCF) of goat semen cryopreserved with 0.25, 0.5, 0.75, 1.0 and 1.25% Equex STM Paste at 5 min (T0), 1 (T1), 2 (T2) and 3 h (T3) after thawing

Parameter	Time	Concentration of Equex STM Paste					
		0%	0.25%	0.50%	0.75%	1.00%	1.25%
ALH	0	7.4±0.5^{ac}	8.3±0.6^{bc}	8.5±0.6^{b}	8.5±0.6^{b}	7.8±0.5^{c}	7.7±0.6^{ac}
(μm)	1	8.0±1.0^{a}	7.6±0.9^{a}	7.6±1.1^{a}	7.7±0.8^{a}	7.8±0.8^{a}	7.9±0.9^{a}
	2	7.4±1.3^{a}	7.2±0.8^{a}	7.2±0.9^{a}	7.3±0.8^{a}	7.3±0.6^{a}	7.4±0.6^{a}
	3	6.6±1.4^{a}	6.9±0.7^{a}	6.9±0.7^{a}	6.7±0.6^{a}	7.0±0.7^{a}	6.9±0.9^{a}
BCF	0	34.8±2.6^{a}	36.0±2.9^{a}	35.6±1.6^{a}	34.1±2.2^{a}	33.7±2.7^{a}	33.3±2.1^{a}
(Hz)	1	36.5±3.1^{a}	37.2±3.7^{a}	37.2±3.4^{a}	36.8±3.1^{a}	34.9±3.7^{a}	34.7±2.8^{a}
	2	35.4±3.8^{ab}	37.6±4.2^{a}	37.3±4.5^{a}	36.9±3.6^{a}	35.1±4.4^{ab}	33.0±3.8^{b}
	3	36.6±3.2^{ab}	38.1±4.2^{a}	37.0±3.8^{ab}	36.2±4.7^{ab}	34.2±3.9^{b}	34.8±3.9^{b}

Within the same parameter, the different superscripts denote values that differ statistically significant ($P<0.05$)

4.5 Discussion

In this study, we examined the effects of freezing extender containing different concentrations of Equex STM Paste for goat semen and found both positive and negative effects of Equex STM Paste on post-thaw spermatozoa quality. The presence of Equex STM Paste in the freezing medium was generally beneficial to goat spermatozoa when compared with the control. Therefore, this finding confirms previous observations on successful semen freezing using freezing extender containing Equex STM Paste in many species (Holt, 2000[a]).

In goat, the presence of egg-yolk coagulation enzymes in the seminal plasma compromises spermatozoa viability during the freezing process especially when egg-yolk based extender is used. Washing and centrifugation of semen prior to adding egg yolk can be performed but these procedures can cause spermatozoa loss and also induce irreversible damage to spermatozoa plasma membrane and acrosome (Corteel, 1981; Azerêdo et al., 2001; Aboagla and Terada, 2004[b]). In practice, egg yolk is frequently used to prepare freezing extender since its compositions protect spermatozoa plasma membrane, DNA fragmentation and lipid peroxidation (Bilodeau et al., 2002), principally via antioxidant properties (Bollwein et al., 2008). Egg yolk also works synergistically with surfactants such as Equex STM Paste and its active component sodium dodecyl sulphate (SDS) (Peterson et al., 2007). Although the mechanisms of Equex STM Paste and SDS in protecting spermatozoa are not completely known, they have been demonstrated to reduce the toxic effects of glycerol and to stabilize the acrosomal membrane during cooling and thawing (Jimenez, 1987). However, it is worth noting that the benefits of Equex STM Paste are more pronounced when egg yolk is added into the freezing extender (Pursel et al., 1978; Dewit et al., 2000; Aboagla and Terada, 2004[a]). This is likely because Equex STM Paste acts as an emulsifier of the egg yolk lipids, thereby making it more accessible to the sperm membrane during cryopreservation (Amann and Pickett, 1987; Buhr and Pettitt, 1996; Dewit et al., 2000). Various concentrations of Equex STM Paste have been added into the freezing extender depending on species, such as 1.5% (v/v) in pig (Buranaamnuay

et al., 2009) and 0.75% (v/v) in rat (Yamashiro et al., 2007). In ruminant, 0.7% (v/v) Equex STM Paste was used in sheep (Akourki et al., 2004) and 0.5-1% (v/v) in bull (Arriola and Foote, 1987). However, these concentrations were not optimal for freezing goat spermatozoa in this study. Freezing extender containing 0.25 and 0.50% Equex STM Paste significantly improved spermatozoa quality compared with other concentrations ($P>0.05$). However, the 0.50% Equex STM Paste was superior. This optimal Equex STM Paste concentration was similar to dog (Rota et al., 1997; Peña and Linde-Forsberg, 2000), cat (Axnér et al., 2004) and horse (Jimenez, 1987).

The beneficial effects of Equex STM Paste were observed at immediately after thawing and during the 3 h incubation after thawing. This 3 h thermal test used in this study because a previous report found that the spermatozoa characteristics 2 to 4 h after thawing are more closely correlated to the fertility of spermatozoa when compared to those measured immediately after thawing (Saacke and White, 1972). The results confirmed previous observations that prolonged incubation of spermatozoa at 37°C adversely affected the quality of spermatozoa when high concentration of Equex STM Paste or SDS were present (goat, Anakkul et al., 2011; El-Kon et al., 2010), (bull, Arriola and Foote, 1987), (mouse, Dewit et al., 2000), (cat, Axnér et al., 2004). Although exact reason for this remain elusive but prolonged exposure of the spermatozoa to Equex STM Paste /SDS may solubilize the spermatozoa's plasma membrane, resulting in the morphological changes and the release of several midpiece enzymes (Churg et al., 1973). Equex STM Paste also has been reported to excessively increase the membrane fluidity due to the increase of free molecules of SDS (Peña and Linde-Forsberg, 2000; Julian et al., 2006). In this study, increasing Equex STM Paste concentrations greater than 0.75% resulted in a sharp reduction in the velocity parameters and had no beneficial effect to the plasma membrane integrity when compared with the lower concentrations. Moreover, we did not observe the detrimental effects of Equex STM Paste on acrosomal membrane integrity irrespective of the concentration of Equex STM Paste. This result differed from previous reports that high concentrations of SDS (active component of Equex STM Paste) negatively affected the acrosomal membrane integrity

(Dewit et al., 2000; El-Kon et al., 2010). The conflicting results of this experiment with current literature may be due to the different freezing technique and extender compositions used.

4.6 Conclusion

Equex STM Paste containing freezing extender improved post-thaw semen quality when compared with non-Equex STM Paste control but the beneficial effects were in a dose dependent manner. The optimal concentration of Equex STM Paste for goat semen freezing was found to be 0.5% (v/v) in glycerol-egg yolk based freezing extender.

CHAPTER V

THE SEASONAL RESPONSE OF SEMEN PRODUCTION AND FREEZABILITY OF CROSSBRED SAANEN GOATS RAISED IN THE TROPICS

5.1 Abstract

Semen characteristics and freezability of eight crossbred Saanen bucks (87.5%) were studied under humid tropical condition (18°Notrh, 100°East). In order to evaluate any seasonal effects, the calendar year was divided into three seasons; winter (November to February), summer (March to June) and rainy season (July to October). The data on the relative humidity, air temperature and daylength were regularly collected. The body weight and scrotal circumference were measured before semen collection. The semen quantity (volume, concentration and total sperm per ejaculate) and quality (motility characteristics, viability, morphology, acrosome integrity, plasma membrane integrity and DNA integrity) were evaluated before and after freeze-thawing. Daylength variation is slight at this latitude (11.00 to 13.16 h), but greater variation exists in temperature (17 to 34°C) and relative humidity (44 to 92%). These were postolated to play a role as seasonal cues and effect semen collection and/or quality. A total of 363 ejaculates were collected and frozen over a 2 year period from 8 bucks. Fresh semen collected in summer and winter was of higher quality. In contrast, the post-thaw semen qualities were not significant different except for motility characteristics and plasma membrane integrity which improved during the winter collections ($P<0.05$). However, the frozen semen quality collected and frozen throughout the year was acceptable for AI (post-thaw motility = 73.12±11.72, 69.36±8.85 and 65.73±15.59 in winter, summer and rainy season, respectively).

5.2 Introduction

Artificial insemination (AI) with frozen-thawed semen allows rapidly genetic improvement and without disease transmission. However, pregnancy rates are lower when synchronized females are artificial inseminated compared to natural breeding. In

order to improve AI results, semen should be collected at an appropriate time using proper management of semen storage and handling (Leboeuf et al., 2000). Although spermatogenesis in the goat can occur throughout the year, the quality and quantity of semen are influenced by several factors (Mohri et al., 1970). Photoperiod has an effect on semen quality especially in higher latitudes (higher than 35°N) (Corteel, 1977; Leboeuf et al., 2000; Barkawi et al., 2006), and the decreasing of day length stimulates reproductive activity (Chemineau et al., 1987; Janett et al., 2003).

In contrast, the influence of day length is minimal in the tropics or subtropics (Chemineau and Xande, 1982; Restall, 1991; Choe et al., 2006). The goats in tropical areas have the ability to breed all year round (Delgadillo et al., 1997). Reproductive ability appears to be modulated by other factors including temperature (Hiroe and Tomizuka, 1966; Yokoki and Ogasa, 1977; Ogebe et al., 1996), humidity (Maina et al., 2006), rainfall (Mukherjee et al., 1953) and nutrition (Gebre, 2007; Zarazaga et al., 2009). The semen production of Nubian, Angora and Boer goats raised in latitudes below 30° do not show any seasonal variations (Grayling and Grobbelaar, 1983; Hibbert et al., 1986). Interestingly, Saanen buck in temperate and tropical climates have been found to be influenced by temperature and photo-period (Mohri et al., 1970; Ahmed et al., 1997; Karagiannidis et al., 2000); however, little information is known about Saanen crossbred offspring. Moreover, the effects of seasonal variation on freezability of goat semen are conflicting (Leboeuf et al., 2000).

The objective of the present study was to determine if Saanen crossbred bucks raised in the tropics maintain seasonal reproductive variation despite minimal changes in day length in the tropical zone. The quality and freezability of semen collected at different seasons within the year were studied.

5.3 Materials and methods

5.3.1 Location of study

The present study was conducted at the CU-Network for Academic Opportunities and Services, Chulalongkorn University, Nan Province, Thailand. It is

situated at the latitude of 18°North and longitude of 100°East within the tropical area. This study was carried out during November in 2009 to October in 2011.

5.3.2 Climatological data, photo period and temperature and relative humidity index (THI)

The calendar year was divided into three seasons, winter (November to February), summer (March to June) and rainy seasons (July to October). The data on relative humidity and the air temperature were supplied by the lascar humidity and temperature USB logger (EL-USB-2, Lascar, United Kingdom), collected every 1 h throughout the experiment. The information on day length was obtained from Thai Meteorological Department. The temperature and humidity index (THI) was calculated using mean temperature (°C) and relative humidity (%) for each season as follows (Thom, 1959; Amundson et al., 2006; Helal et al., 2010).

THI = (0.8 x Mean temp) + (RH/100) x (Mean temp-14.4) + 46.4

5.3.3 Animals

The eight post-pubertal crossbred Saanen bucks (87.5%) approximately 1 year of age were used for the 24-month study (following the same bucks). These bucks were maintained in free stall housing under preventive and clinical veterinary care. The diet consisted of a concentrate (14% protein) with grasses, mineral blocks and water *ad libitum*. Range grazing was available all year.

5.3.4 Semen collection, measurement of scrotal circumference and body weight

Each buck was weighed and scrotal circumference measured before semen collection. The maximum scrotal circumference was determined using a flexible measuring tape. Semen was collected from each buck by artificial vagina every two weeks at 9 a.m. over a two years period. The 49 semen collections per animal were possible. Only semen samples with adequate motility (>70%) and sperm concentration (>1,500 x 10^6 spermatozoa/ml) were pooled together for further process.

5.3.5 Semen freezing and thawing procedures

The basic semen extender in this experiment was Tris (hydroxymethyl-aminomethane)-citric acid-fructose (TCF) solution, which contained 250.0 mM Tris, 90.0 mM citric acid and 70.0 mM fructose. The solution was adjusted to pH 7.0-7.2. TCF (without egg yolk and glycerol) was used as washing solution for removing seminal plasma. Ten percentages of egg yolk, glycerol (v/v) (Sigma, Steinheim, Germany), and 0.5% Equex STM paste (Nova Chemical Sales Inc., Scituate, MA, USA) were added in TCF for freezing extenders. The pooled semen samples were placed in a centrifuge tube, diluted with TCF (1:9), and washed by centrifugation at 910 x g for 10 min at 28 °C. The supernatant was discarded and the semen sediment was diluted with freezing extender, and adjusted to a concentration of 100 x 10^6 spermatozoa/straw (400 x 10^6 spermatozoa/ml). The diluted semen was equilibrated at 4°C for 4 h. After equilibration, the samples were loaded into 0.25 ml French mini straws (Minitüb, Landshut, Germany) and placed horizontally approximately 4 cm above the liquid nitrogen level for 10 min in a styrofoam box, before being plunged into the liquid nitrogen. The samples were thawed by placing in a 37°C water bath for 30 sec.

5.3.6 Sperm evaluation

The ejaculated semen was evaluated for the volume and pH (pH-Indicator paper, Neutralit®, Merck, Germany). Mass movement (0-4) and percentages of motility were subjectively estimated under light microscope (100 x) within 10 min. Sperm concentration was calculated using hemocytometer slide (Neubauer, Boeco, Germany). The viability, head morphology, tail morphology, acrosome integrity, DNA integrity and plasma membrane integrity, were assessed in fresh semen of each buck and post-thaw samples. All assessments of the post-thaw samples were evaluated after incubation (37°C) at 5 min, 1, 2 and 3 h (T0, T1, T2, and T3, respectively).

Motility characteristics were evaluated by computer-assisted sperm analysis (CASA, IVOS model 12.3, Hamilton-Thorne Biosciences, Bevery, MA, USA) only in post-thaw samples. The CASA setting was performed following the manufacturer's recommendation. The frame rate for semen analysis was set up at 60 Hz (Sundararaman and Edwin, 2008; Bucak et al., 2010). After thawing, samples were diluted with

phosphate buffered saline (1: 9), and then 10 µl of the diluted sample was loaded in 2X-CEL chambers slide (Hamilton-Thorne, Inc., MA, USA), which was pre-warmed to 37 °C. The samples were analyzed for total motility (MOT, %), progressive motility (PMOT, %), average path velocity (VAP, µm /s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz). The mean values of three randomly selected fields were used for statistical analysis.

For viability and head morphology assessment, spermatozoa were stained with eosin-aniline blue stain and William's stain, respectively. They were examined microscopically (x1000). A total of 200 spermatozoa fixed in 4% (w/v) formaldehyde solution in phosphate buffer saline (PBS) were evaluated for tail morphology under a phase contrast microscope (400x) (CX41RF; Olympus, Tokyo, Japan). A fluorescein isothiocyanated peanut agglutinin (FITC-PNA) stain was used to evaluate the acrosome integrity as previously described by Axnér et al. (2004), with slightly modifications (Anakkul et al., 2011). DNA integrity was determined using a metachromatic dye (acridine orange stain; Tejada et al., 1984). The technique was modified as briefly described, the spermatozoa were smeared on a glass slide and allowed to air-dry. They were subsequently fixed overnight in freshly prepared methanol: glacial acetic acid (3:1), and then air-dried. The slides were stained with acridine orange (AO; Sigma-Aldrich, St. Louis, USA) staining solution (0.25%, w/v) for 5 min at room temperature. At least 200 spermatozoa for FITC-PNA stain or 500 spermatozoa for acridine orange stain per sample were evaluated using a fluorescent microscope (BX51; Olympus, Shinjuku, Japan). The hypoosmotic swelling test (HOST: 100 mOsM/kg) was used for testing plasma membrane integrity (Nur et al., 2005).

5.3.7 Statistical analysis

The results are expressed as mean±SE or mean±SD (climatological data of Fig.3). Climatological data and THI were evaluated using the general linear model (GLM), while sperm data were examined using the repeated measure statement of the MIXED procedure (season, animal and year as fixed variable) of the Statistical Analysis

Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). The values with $P<0.05$ were considered statistically significant.

5.4 Results

5.4.1 Climatological data, day length and temperature and relative humidity index (THI)

The mean environmental temperature and relative humidity during the experimental period ranged between 17 to 34°C and 44 to 92%, respectively. The temperature was lowest in winter ($P<0.05$).The relative humidity was greatest during the rainy season and was the least during the summer. The day length was almost constant, approximately 12.13 h throughout the year. Similar to relative humidity, THI mean was higher in rainy season but reached its minimum during the winter ($P<0.05$). The shortest and longest days corresponded to 11.00 h in the winter and 13.16 h in the summer, respectively. The climatological data, THI and daylight hour information is summarized in Table 10 and Fig. 3. Similar seasonal pattern was observed during the experimental period.

Table 10 Climatological data (mean±SE) for years 2009-2011.

Season	Month	Temperature (°C)			Relative humidity (%)			THI	Day length (h)
		Mean	Min	Max	Mean	Min	Max		
Winter	Nov-Feb	22.81^{a}	17.96^{a}	30.93^{a}	75.44^{a}	47.64^{a}	91.23^{a}	70.95^{a}	11.14
Summer	Mar-Jun	27.97^{b}	22.77^{b}	34.79^{b}	69.72^{b}	47.11^{a}	86.98^{b}	78.64^{b}	12.42
Rainy	Jul-Oct	27.38^{c}	24.03^{b}	33.26^{c}	83.56^{c}	62.78^{b}	94.12^{c}	79.17^{c}	12.28
SE		0.15	0.54	0.20	0.75	0.52	0.33	0.22	0.36

Different superscripts $^{(a,b)}$ indicate significant differences within column ($P < 0.05$).

5.4.2 Seasonal effect on body weight, scrotal circumference (SC), number of ejaculations and fresh semen characteristics

The body weight, SC and characteristics of ejaculated semen from eight crossbred Saanen bucks collected during three distinct seasons are presented in Table 11. The body weight and SC of the bucks were influenced by season with the greater weight and SC occurring during the summer months ($P<0.05$).

The total numbers of ejaculates from each buck during each of the three seasons are shown in Table 12. A higher percentage of bucks that failed to ejaculate was greater in the rainy season followed by the winter and then the summer. Semen quality, volume and concentration, were also affected by season. Semen volume and total sperm per ejaculation were greater during rainy season and lowest in the winter (Fig. 3). Semen concentration ($P<0.05$) and pH followed the opposite trend with the highest values occurring in the winter. Significant seasonal differennce among seasons were found in most of the semen qualities examined, with the exception of acrosome membrane integrity, which remained constant throughout the year. The quality of semen appeared higher in the summer exhibiting superior values for all semen quality measured except for plasma membrane integrity, which was at its lowest value in the summer season. The highest motility was found in rainy season but a decreasing trend of mass movement, viability, normal head morphology, acrosome membrane integrity, and DNA integrity were observed.

Table 11 The value (mean±SE) of body weight, scrotal circumference and semen characteristics of crossbred Saanen bucks in three distinct seasons

Parameter	Unit	Winter	Summer	Rainy	SE
Weight and scrotal circumference					
Body weight	kg	53.53^{a}	59.61^{b}	56.48^{c}	0.69
Scrotal circumference	cm	24.15^{a}	25.87^{b}	24.97^{c}	0.15
Semen characteristic					
pH	-	7.09^{a}	7.00^{ab}	6.88^{b}	0.55
Volume	ml	0.67^{a}	0.74^{a}	0.86^{b}	0.37
Concentration	x10^{9}sperm/ml	4.57^{a}	4.44^{ab}	3.93^{b}	0.22
Total sperm	x10^{9}sperm	3.36^{a}	3.51^{a}	3.6^{a}	0.29
Mass movement	0-4	3.28^{a}	3.13^{a}	2.75^{b}	0.07
Motiliy	%	70.47^{a}	72.56^{ab}	73.15^{b}	0.99
Viability	%	79.02^{a}	81.63^{b}	78.18^{a}	1.21
Normal tail	%	93.16^{a}	95.04^{b}	94.45^{ab}	0.69
Normal head	%	99.56^{a}	99.68^{b}	99.32^{c}	0.09
Acrosome intact	%	99.82^{a}	99.91^{a}	99.74^{a}	1.56
HOST positive	%	45.53^{a}	40.67^{b}	43.19^{ab}	1.74
DNA integrity	%	99.82^{a}	99.91^{b}	99.74^{a}	0.04

Within the same parameter, the different superscripts denote values that differ statistically significant ($P<0.05$)

Table 12 The distribution of ejaculates collected from eight crossbred Saanen bucks between November 2009 and October 2011

Variable	N*									Total
		I	II	III	IV	V	VI	VII	VIII	%
Ejaculates per season										
Winter	17	16	16	16	15	15	15	15	16	91.18
Summer	16	16	16	16	16	16	13	16	13	95.31
Rainy	16	16	16	15	13	15	14	15	12	90.63
ejaculates .No	49	48	48	47	44	46	42	46	42	

*N - the number of times that semen collections were performed in each season

5.4.3 Seasonal effect on freezability

The effect of season was clearly seen on most of motility characteristics (Fig. 4) and plasma membrane integrity (Table 13), which the greatest motility pattern and higher percentage of swelling tail in HOST was found in winter. However, ALH (5.74-8.43 μm), one of the motility characteristics, was not found any significant difference among seasons. Seasonal variation in viability and acrosome integrity was not so strong. However, these parameters were also greater during the winter season ($P>0.05$; Table 12). The morphology and DNA integrity were not affected by seasonal variation. All evaluated mean values were higher than 98.84, 91.56 and 99.80% for normal head morphology, normal tail morphology and DNA integrity, respectively.

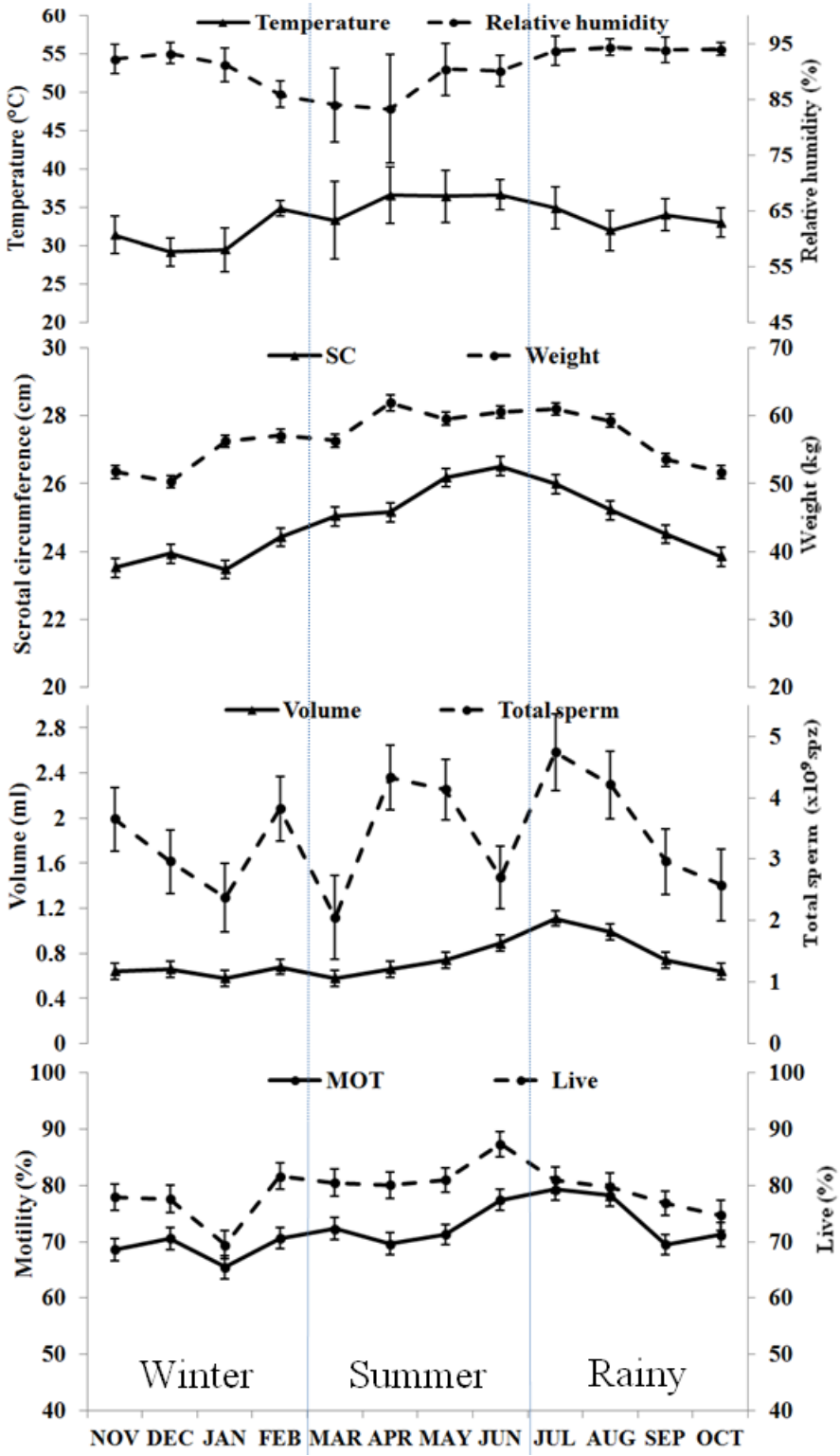

Figure 3 Climatological data (mean±SD), body weight (mean±SE), scrotal circumference (mean±SE) and semen production (mean±SE) of bucks evaluated in each month during the experimental years (2009-2011).

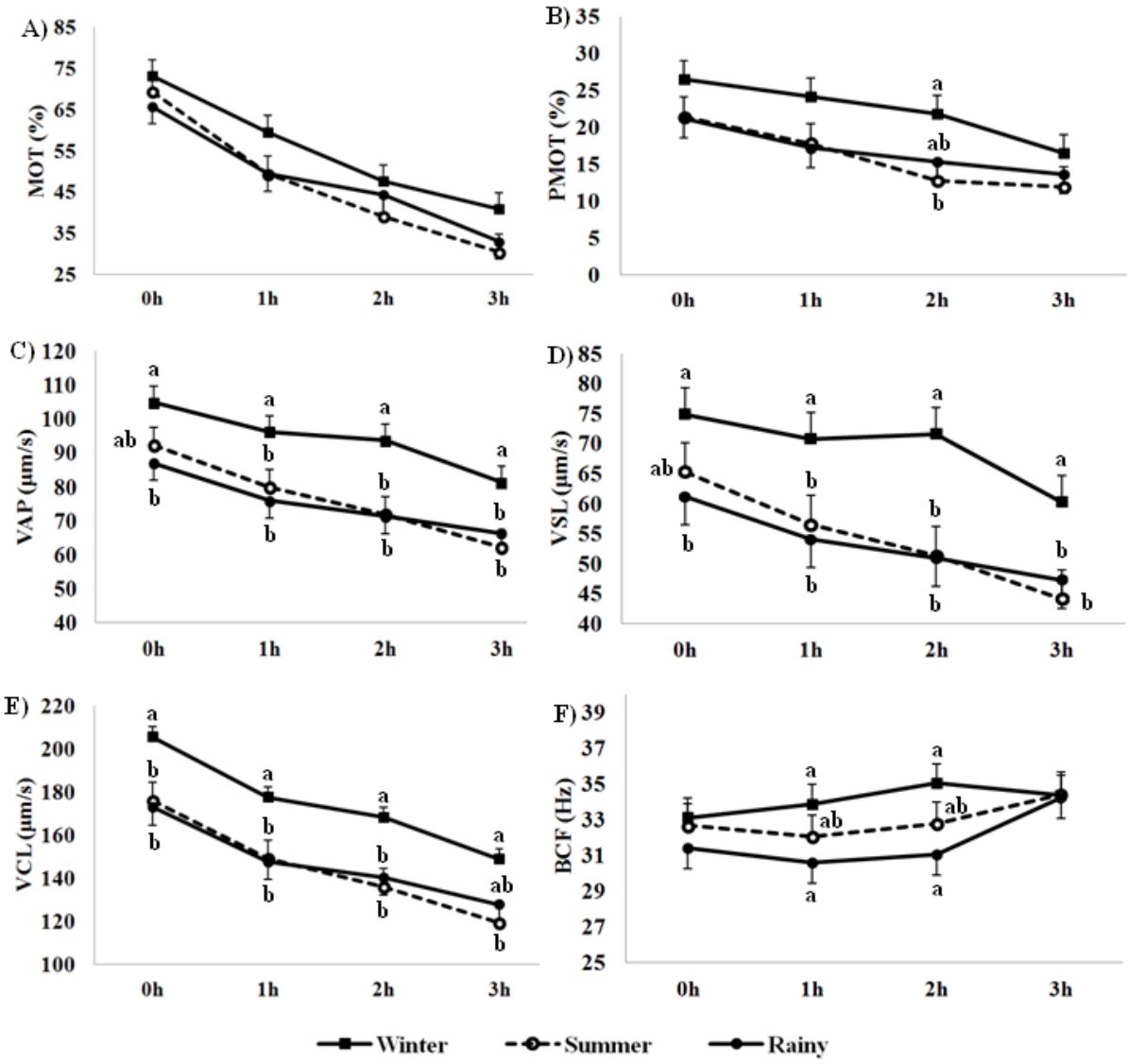

Figure 4 Motility characteristics pattern (mean±SE) of frozen-thawed spermatozoa at 0, 1, 2 and 3h after thawing in each season. A: total motility (MOT, %), B: progressive motility (PMOT, %), C: average path velocity (VAP, μm/s), D: straight line velocity (VSL, μm/s), E: curvilinear velocity (VCL, μm/s) and F: beat cross-frequency (BCF, Hz)

Table 13 The percentages of viability, acrosome integrity and plasma membrane integrity of goat semen after thawing at 0, 1, 2 and 3 h in each season

Time	Viability (%)			Acrosome integrity (%)			HOST* positive (%)		
(h)	Winter	Summer	Rainy	Winter	Summer	Rainy	Winter	Summer	Rainy
0	67.97	67.46	67.97	89.85	90.00	91.47	49.32^{a}	41.57^{b}	42.58^{ab}
1	58.94	55.25	55.40	86.94	85.57	89.73	36.62^{a}	31.46^{a}	30.35^{a}
2	54.82	48.82	51.27	83.44	81.50	84.73	32.74^{a}	25.54^{b}	26.50^{ab}
3	49.85	44.29	47.84	78.59	77.93	83.64	28.53^{a}	23.25^{a}	24.46^{a}
SE	3.18	3.51	3.39	1.89	2.08	2.01	2.32	2.57	2.66

Within the same parameter, the different superscripts denote values (^{a,b}) that differ statistically significant ($P<0.05$) between column

*HOST-Hypoosmotic swelling test. Test for membrane integrity.

0=5 minutes after thawing

5.5 Discussion

The crossbred Saanen bucks born and raised under the humid tropical conditions still show seasonal variation on semen production and freezability. Overall, the fresh semen collected in summer and winter provided a higher in quality than rainy season. For frozen semen, the post-thaw semen qualities were not significant different except for motility characteristics and plasma membrane integrity which showing their best in winter. However, the frozen semen quality was acceptable for AI using during all seasons of the year.

Many studies reported about a seasonal breeder in goat raised in higher latitudes (higher than 35°N) during short day period (Corteel, 1977; Chemineau et al., 1987; Leboeuf et al., 2000; Barkawi et al., 2006). However, our results revealed that semen productions of crossbred Saanen still maintained their seasonal effect in lower latitude (18°N) despite of a little variation in day length. Our finding coincided with the findings of Ahmed et al. (1997) who established the seasonal change on reproductive

behavior in Sudan (15°30´N, 32°E).The temperature and humidity were hypothesized to play an important role under this humid tropical conditions (Ogebe et al., 1996).

The highest in temperature during summer time have been demonstrated to limit on physiological responses and semen quality in goats under subtropical and tropical climates (Ogebe et al., 1996), but the fresh semen obtained in summer and winter provided a better quality than in rainy. This may be resulted from a lower of relative humidity in summer since relative humidity was reported to be influenced on goat reproduction (Maina et al., 2006).

The testicular size has been shown to be a reliable measurement of the reproductive growth status and spermatogenic capacity more than body weight (Daudu, 1984; Delgadillo et al., 1991; Ahmad and Noakes, 1995). Due to the fact that the semen production rate per unit weight is constant, the larger testes produced more semen (Gherardi et al., 1980; Preston et al., 2001). However; the total number of spermatozoa per ejaculate was not higher in summer despite of the significant larger of SC was presented. This might be the influence of body weight which also significant increase in this season. The largest of mean SC presented in summer was similar to previous reports in British (Ahmad and Noakes, 1995), Damascus (Al-Ghalban et al., 2004) and Zaraibi goats (Barkawi et al., 2006). The greater in SC has been related to seminal characteristics which were supported in this study by a greater quality in viability ($P<0.05$), normal sperm morphology ($P<0.05$), DNA integrity ($P<0.05$) and acrosome integrity, similar to Rayini goats reported in Iran (Zamiri and Heidari, 2006).

Without vary seasonally on the total number of spermatozoa per ejaculate, the seasonal variations were found on other semen quantity parameters (volume and concentration) as reported in ram (Simplício et al., 1982). The total sperm per ejaculation is more influenced by semen volume compared with a concentration of semen which was in agreement with other studies in sheep (Cupps et al., 1960) and goats (Karagiannidis et al., 2000; Catunda et al., 2011).

The information about semen quality collected in rainy season is variable. A higher semen quality in rainy was reported due to higher rainy season feed (Carmenate

and Gamcik, 1982) that provided positive influence on nutrition in goat (Zarazaga et al., 2009) and ram (Martin and Walkden-Brown, 1995). This effect has not been seen in our experiment which in agreement with another report in sheep (Gordon, 1997). From our results, a lot of semen parameters, except for volume, total sperm per ejaculate and motility were significant lower in rainy. The percentage of time that bucks refuse to ejaculate was also highest in rainy. The highest in THI which fell in the range of severe stress (>78 to 89; Armstrong, 1994) of rainy season can result in heat stress (Suriyasomboon et al., 2004) which was explained to be a cause of some negative effect on sperm production (Mukherjee et al., 1953; King, 1993). The decreasing of motility and an increasing of percentage of abnormal spermatozoa have been reported in pig due to heat stress and high humidity (Murase et al., 2007) because heat stress affects on elongated and round spermatids, and late spermatocytes in the spermatogenesis (Cameron and Blackshaw, 1980).

The significant lowest mean THI was observed in winter ($P<0.05$). The highest concentrations together with the lowest of semen volume were found in this season. Although the other characteristics of fresh semen collected in winter were not dramatically superior to other seasons, the post-thaw qualities were clearly shown their best in winter, especially for motility characteristics and plasma membrane integrity.

In conclusion, despite of the difference on semen production among season, the quality of fresh semen throughout the year provided an accepted quality after freezing-thawing in every season. The best motility characteristics and plasma membrane intact of post-thaw spermatozoa were shown in winter.

CHAPTER VI

SPERM DISTRIBUTION AND FERTILIZATION IN UNILATERAL AND BILATERAL LAPAROSCOPIC ARTIFICIAL INSEMINATION OF FROZEN GOAT SEMEN

6.1 Abstract

Generally, laparoscopic artificial insemination (LAI) provides a higher success rate than that of cervical insemination in goat. Our objectives were to evaluate the distribution of frozen-thawed spermatozoa after LAI and to compare the effect of sperm numbers and deposition sites (unilateral and bilateral sites) on pregnancy rate. In experiment 1, the frozen-thawed spermatozoa were stained with CellTracker™ Green CMFDA (CT-Green) or CellTracker™ Red CMPTX (CT-Red), and then evaluated *in vitro* for viability and motility at 0, 3, 6 and 9 h to test the probable toxicity of the dyes. Experiment 2, the CT-Green and CT-Red labeled spermatozoa were laparoscopically deposited into the left and right uterine horns, respectively. After ovariohysterectomy in 6 h later, the total number of green and red colored spermatozoa were collected and counted via tissue section, sperm flushing and collection of oviduct contents. Experiment 3 was designed to test the pregnancy in 120 does following LAI using different numbers of spermatozoa (60 and 120 $x10^6$ spermatozoa per LAI) and deposition sites. The results revealed that the fluorochromes used in this study did not impair the sperm motility and viability. The frozen-thawed goat spermatozoa can be transuterine migrated following laparoscopic insemination and the total number of 60 $x10^6$ spermatozoa inseminated into one uterine horn without ovarian observation can efficiently be used for laparoscopically intrauterine insemination

6.2 Introduction

Frozen-thawed semen is commonly used for genetic improvement of livestock animals. However, this semen must be deposited close-by the site of fertilization (i.e. into the uterus) in order to increase pregnancy rate after AI. In goat, laparoscopic

artificial insemination (LAI) provides a higher pregnancy rate than that of cervical AI (Ritar et al.,1990 ; Sohnrey and Holtz, 2005). However, the conception rates of LAI are variable from 20 to 70% (Ritar et al., 1987; Goonewardene et al., 1996; Martínez-Rojero et al., 2007), largely depending on quantity and quality of spermatozoa being inseminated (Rodriquez et al., 1988). LAI is also favorable for commercial proposes because this technique allows using lower numbers of spermatozoa than that of traditionally transcervical insemination (Maxwell et al., 1984). However, pregnancy rate is decreased when insufficient sperm numbers are used for AI (Hunter et al., 1982). The numbers of inseminated spermatozoa are therefore required to be optimized in order to achieve the highest pregnancy rates. To date, a large variability of insemination doses (5-200 x 10^6) has been reported for frozen-thawed goat spermatozoa depending largely on the AI technique employed and spermatozoa quality (Ritar et al., 1990; Baril et al., 1996; Buckrell, 1997; Sohnrey and Holtz, 2005; Martínez-Rojero et al., 2007). However, most researches on dose and site of insemination (ipsilateral or contralateral site of an ovulation) have been performed in sheep, and little is known in goats (Ehling et al., 2003; Martínez-Rojero et al., 2007; Kulaksiz and Daşkin, 2012). Practically, LAI is more frequently used for frozen-thawed semen compared with cervical insemination due to the high pregnancy rate after LAI. It seems likely that the pregnancy rate is high when semen is deposited into both uterine horns (Maxwell, 1986). To perform this, the spermatozoa are halved and deposited into each uterine horn approximately at midway between the uterine body and utero-tubal junction (UTJ) (Evans et al., 1987; Ritar et al., 1990; Baril et al., 1996; Martínez-Rojero et al., 2007; Kulaksiz and Daşkin, 2012). Although this technique yields high pregnancy rate, this double-horn insemination is time consuming and labor intensive. Thus, single-uterine horn insemination without ovarian examination via LAI would be simpler and more practical for goat industry. However, this procedure will be successful when deposited spermatozoa can be transported to both sides of reproductive tracts. It is therefore essential to study the sperm transport within the reproductive tracts of female goats following LAI. Several studies also indicated that the spermatozoa can be migrated throughout the uterus and

oviducts even the insemination was performed into only one uterine horn of dog (Fukushima et al., 2010), pig (Tummaruk et al., 2010) and sheep (Eppleston et al., 1994; Correa et al., 1994). However, the sperm distribution following LAI in goat remains unknown especially when frozen-thawed semen is used. To identify the sperm distribution in the female genital tracts, several techniques have been used, for examples, uterine and oviductal flushing, mucosal imprint, histology and supravital staining with non-toxic fluorochromes (Druart et al., 2009; King et al., 2002).

The objectives of this study were therefore to evaluate sperm distribution after intrauterine insemination with frozen-thawed spermatozoa previously labeled with fluorochromes, as well as, to study the effects of sperm numbers and insemination sites on pregnancy rates following laparoscopic insemination in goats.

6.3 Materials and methods

6.3.1 Experimental animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC), Chulalongkorn University (Approval No. 11310030). Post pubertal crossbreed Saanen (75%) goats, aged between 2 and 4 years old, were used. The animal facility was located at the CU-network for Academic Opportunities and Services, Chulalongkorn University, Nan Province, Thailand (latitude 18°N and longitude 100°E). They were maintained in a semi-intensive system under preventive and clinical veterinary care. The goats were daily fed with concentrates (14% protein, w/w), *ad libitum* of grass with free access to mineral blocks and water.

6.3.2 Experimental design

Experiment 1: Post-thaw quality of frozen-thawed spermatozoa after supravital staining

Semen was collected from eight bucks, pooled together, and then frozen. The spermatozoa were thawed and immediately stained with CellTracker™ Green CMFDA (CT-Green) or CellTracker™ Red CMPTX (CT-Red) in order to test the probable toxicity of the supravital dyes. After staining, the spermatozoa were then assessed at 0, 3, 6 and 9 h for sperm quality, in terms of motility and viability as described in previous chapter.

Experiment 2: Distribution of frozen-thawed spermatozoa following laparoscopic insemination

To study on the distribution of sperm *in vivo*, the spermatozoa were first labeled either with CellTracker™ Green CMFDA (CT-Green) or with CellTracker™ Red CMPTX (CT-Red) in order to distinguish the distribution of spermatozoa from different insemination sites. The green and red labeled spermatozoa ($30x10^6$ spermatozoa in 0.125 ml) were deposited into the left or right uterine horn, respectively. At 6 h after LAI, the does (n=4) were ovariohysterectomized (OVH). The spermatozoa were collected or counted separately from each side of uterus and oviducts via 3 techniques (tissue section, sperm flushing and collection of oviduct contents). The spermatozoa positive to each fluorescent dye were counted in each particular part of oviducts under fluorescent microscope.

Experiment 3: Effects of sperm numbers and insemination technique on pregnancy rate

A total of 120 crossbred Saanen were used in this study. All goats were divided into four groups (30 does in each group). The does were LAI inseminated with different sperm numbers ($60x10^6$ and $120x10^6$) using unilateral (group 2 and 4) or bilateral (group 1 and 3) intrauterine insemination (Table 13). These insemination regimens were performed either with or without ovarian observation. If the ovaries were observed, the insemination was performed at ipsilateral site of the presence of graafian follicle. The pregnancy rate was determined on 45 days after LAI by ultrasonography.

6.3.3 Semen collection, cryopreservation and thawing

Goat spermatozoa from eight bucks were collected using artificial vagina. After seminal plasma removal, the ejaculates were pooled and diluted with Tris-citric acid-fructose (TCF) extender supplemented with 0.5% (v/v) Equex STM Paste (Nova Chemical Sales Inc., Scituate, USA), 10% (v/v) glycerol (Sigma-Aldrich St. Louis, USA) and 10% (v/v) egg yolk. The spermatozoa were frozen by styrofoam box method as previously described (Anakkul et al., 2011). In brief, the diluted semen ($240x10^6$spermatozoa/ml) was equilibrated at 4°C for 4 h and then loaded into 0.25 ml

French mini straws (Minitüb, Landshut, Germany). The straws were placed horizontally in liquid nitrogen vapor at 4 cm above the liquid nitrogen level for 10 min and then plunged into liquid nitrogen. When required, the spermatozoa were thawed by placing the straw in a 37 °C water bath for 30 sec.

6.3.4 Supravital dye labeling and assessment of sperm quality

All fluorescent probes were purchased from Invitrogen (Eugene, Oregon, USA). To prepare the stock solutions of CellTracker™ Green CMFDA (CT-Green) and CellTracker™ Red CMPTX (CT-Red), the lyophilized products were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA) to a final concentration of 10 mM. The stock solutions were further diluted in 10% egg yolk-TCF to a working concentration of 25 µM.

After thawing, the spermatozoa were evaluated for motility. They were further diluted with 10% egg yolk-TCF at a ratio 1:5 and then centrifuged at 940 x g for 10 min at room temperature (26°C) to remove glycerol and Equex STM Paste that may be toxic to the spermatozoa (Peña and Linde-Forsberg, 2000; Anakkul et al., 2011). The supernatant was discarded, and the spermatozoa were then divided into three equal aliquots. The first aliquot was resuspended with 500 µl of 10% egg yolk-TCF as a control group, while the other two aliquots were added with CT-Green (25 µM) or CT-Red (25 µM) in 500 µl of 10% egg yolk-TCF. After 1 h incubation, the spermatozoa were centrifuged to remove excessive dye. The spermatozoa concentration was adjusted to 60 x 10^6 spermatozoa/0.25 ml with 10% egg yolk-TCF extender and then incubated at 37°C for longevity test. The spermatozoa were examined at 0, 3, 6 and 9 h for motility and viability (three replicates) using a phase contrast microscope (100x) (CX41RF; Olympus, Tokyo, Japan) and eosin-aniline blue staining, respectively. The fluorochrome intensity was also additionally examined under a fluorescent microscope (BX51; Olympus, Shinjuku, Japan) using excitation/emission of 492/517 nm and 577/602 nm for CT-Green and CT-Red, respectively.

6.3.5 Estrus synchronization and ovariohysterectomy (OVH)

The does were synchronized using an intravaginal progesterone sponge, Sincro-gest sponges® (65 mg of Medroxyprogesterone; Laboratorios Ovejero, Leon, Spain), for

13 days with a single injection of 400 IU pregnant mare serum gonadotropin (Sincro-gest PMSG®; Laboratorios Ovejero, Leon, Spain) before sponge removal. Estrus was detected with an apronized buck every 12 h after sponge removal. An injection of 300 IU human chorionic gonadotropin (hCG; Chorulon®, Intervet Schering-Plough Animal Health, The Netherlands) was done when estrus sign was first detected in each doe, and LAI was performed once at 21 h later. The goats were free of feed and water for 24 h before the operation (Mobini et al., 2002). The LAI was performed in four does as described in brief as follows. The does were anesthetized with an intravenous injection of xylazine HCl (0.1 mg/kg) and ketamine HCl (4 mg/kg). The analgesic drugs (4 mg/kg of phenylbutazone) and antibiotic (20,000 IU/kg of penicillin-streptomycin LA) was also given (i.m.). The goats were later placed in dorsal recumbency, with the head tilted down position (Mobini et al., 2002).

The labeled spermatozoa were inseminated into the uterine horn at half way between the UTJ and the bifurcation via dwelling intravenous catheter (Maxwell, 1986; Ritar et al., 1990). The CT-Green and CT-Red spermatozoa (30×10^6 spermatozoa in 0.125 ml) were deposited to left and right uterine horns, respectively. At 6 h after insemination, the does were OVH under general anesthesia as described above. The ovulation was defined from the presence of corpus haemorrhagicum (CH) observed.

6.3.6 **Sperm recovery and assessment of sperm transport**

After OVH, the reproductive tracts were dissected and cumulative numbers of spermatozoa were collected separately from each side by three techniques including tissue section, sperm flushing and collection of luminal contents of oviduct. The oviduct was divided into three segments which were ampulla, middle part and isthmus (together with UTJ). Each segment was cut (about 1 cm) at the proximal end for tissue section. The remaining oviducts were individually flushed with phosphate buffered saline (PBS) supplemented with 0.1% (w/v) bovine serum albumin to recover the free floating spermatozoa, and the luminal contents of the flushed oviducts were collected by gentle squeezing the oviduct (Latham and Westhusin, 2000; Gualtieri et al., 2005). For the uterus, the flushing was similarly performed and the tissues were collected from middle

and proximal parts of each side of uterine horns but without collection of the luminal contents.

The recovered spermatozoa from each side of oviducts and uterine horns were evaluated at a magnification x 200-400 under a fluorescent microscope (BX51; Olympus, Shinjuku, Japan).

6.3.7 Cryosection of the oviduct and uterus

The middle (uterine horn) and proximal ends (uterine horn and three segments of oviduct), about 1 cm in size of each segment, were fixed in 4% (w/v) formaldehyde in PBS overnight. All tissue segments were washed with PBS and then incubated in 20% (w/v) sucrose solution for dehydration. The tissues were transversely cryo-sectioned at a thickness of 10 μm. The presence of CT-Green and CT-Red-labeled spermatozoa in each uterine horn and oviduct section was determined under fluorescent microscopy.

6.3.8 Laparoscopic artificial insemination (LAI)

The does were estrus synchronized as before being inseminated and OVH in experiment 2. After an estrus was detected, LAI was performed at 21 h later under general anesthesia. The post-thaw spermatozoa were inseminated into the uterine lumen in the middle of uterine horn (half way between the UTJ and the bifurcation) under an observation via a direct view 5-mm laparoscope (Schölly, Denzlingen, Germany). The total of 120 does were LAI with a difference numbers of spermatozoa and sites of insemination (Table 14).

The pregnancy was diagnosed at 45 d after insemination by real-time B mode cutaneous ultrasonography (5 MHz) (HS-2000, Honda Electronics Co., Ltd., Aichi, Japan).

Table 14 The four groups of does inseminated with different protocols

Group	Subgroup	Ovarian observation	n	Insemination dose (x10^6 spermatozoa)	
				First horn	Second horn
1	1.1	Yes	15	30	30
	1.2	No	15	30	30
2	2.1	Yes	15	60	NA
	2.2	No	15	60	NA
3	3.1	Yes	15	60	60
	3.2	No	15	60	60
4	4.1	Yes	15	120	NA
	4.2	No	15	120	NA

NA (not applicable): no spermatozoa deposited into the uterine horn.

6.3.9 Statistical analysis

The semen parameters are presented as mean±SD. The comparison among groups (control, CT-Green and CT-Red) and evaluated time were analyzed by general linear model procedure (GLM). The numbers of recovered spermatozoa are presented as mean±SD. Chi-squared test was used to compare the pregnancy rates. The statistical analysis was performed using SAS software (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). The values were significantly different when $P<0.05$.

6.4 Results

6.4.1 Experiment 1: Post-thaw quality of frozen-thawed spermatozoaf after supravital staining

After thawing, sperm motility and viability were 76.67±2.89 and 76.17±2.75, respectively. The post-thaw motility and viability gradually decreased along the incubation times. Both motility and viability significant reduced after 6 h post-incubation in all group ($P<0.05$). Supravital staining used in this study impaired neither spermatozoa quality nor longevity when compared between the two fluorochromes with

non-staining control (Table 15). The sperm motility and viability were well maintained to be higher than 50% when examined at 6 h post-staining.

Table 15 The motility and viability (mean±SD) of spermatozoa stained with CT-Green and CT-Red

Incubation	Motility (%)			Viability (%)		
time	Control	CT-Green	CT-Red	Control	CT-Green	CT-Red
0 h	68.33±2.89^{a}	68.33±2.89^{a}	68.33±2.89^{a}	68.17±0.29^{a}	68.33±2.93^{a}	67.50±3.50^{a}
3 h	65.00±0.00^{a}	65.00±0.00^{ab}	66.67±2.89^{ab}	61.67±2.08^{b}	63.33±2.25^{ab}	67.50±3.91^{a}
6 h	55.00±8.66^{b}	56.67±5.77^{b}	58.33±7.64^{b}	57.67±4.48^{b}	60.50±5.77^{b}	58.67±5.53^{b}
9 h	25.00±8.66^{c}	25.00±8.66^{c}	23.33±7.64^{c}	49.00±1.32^{c}	51.83±1.26^{c}	54.5±3.50^{b}

Within a column, different superscripts denote values that differ statistically significant ($P<0.05$).

6.4.2 Experiment 2: Distribution of frozen-thawed spermatozoa following laparoscopic insemination

All four does responded to estrus synchronization program with the presence of large follicle when LAI was performed. Estrus sign was first detected at 58.13±8.34 h after sponge removal. At the time of OVH, ovulation had occurred in three of the four does. The remaining doe had a large follicle on the ovary.

The results from cryosection indicated that unilaterally inseminated spermatozoa with $30x10^{6}$ spermatozoa in 0.125 ml could distribute through the entire uterine lumen since both CT-Green (left insemination) and CT-Red labeled spermatozoa (right insemination) were found in both sides (Fig. 5). Similarly, spermatozoa were recovered from all segments of uterine and oviductal flushing (Fig. 6). The numbers of recovered spermatozoa per uterine and oviduct segments are shown in Table 16. The majority of recovered spermatozoa were obtained from the uterus with a large variation in the numbers of recovered spermatozoa among the animals. However, spermatozoa were not presented in the contents from middle and ampulla parts. These results were supported by the finding that most spermatozoa observed in the cryosection samples

were located, individually or in groups, in the crypts of the uterine horns and isthmus-UTJ (Figs. 7, 8), but not in the middle and ampulla parts of the oviduct.

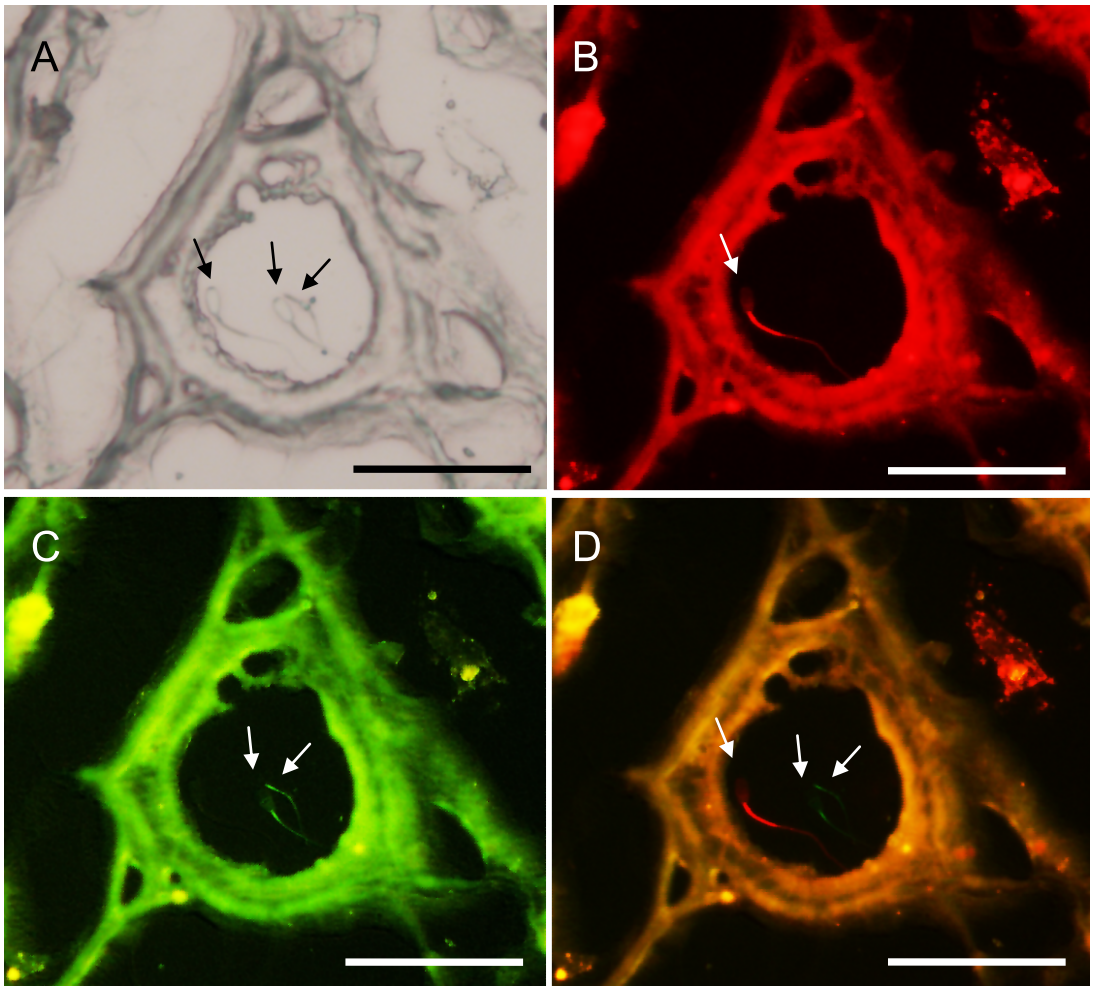

Figure 5 Photomicrographs of left uterine horn in bright field (A) which both CT-Red (B) and CT- Green (C) labeled spermatozoa presented in the same field, as well as the merged image represented CT-Green and CT-Red (D). The arrows indicated spermatozoa. Scale bars represent 50 μm.

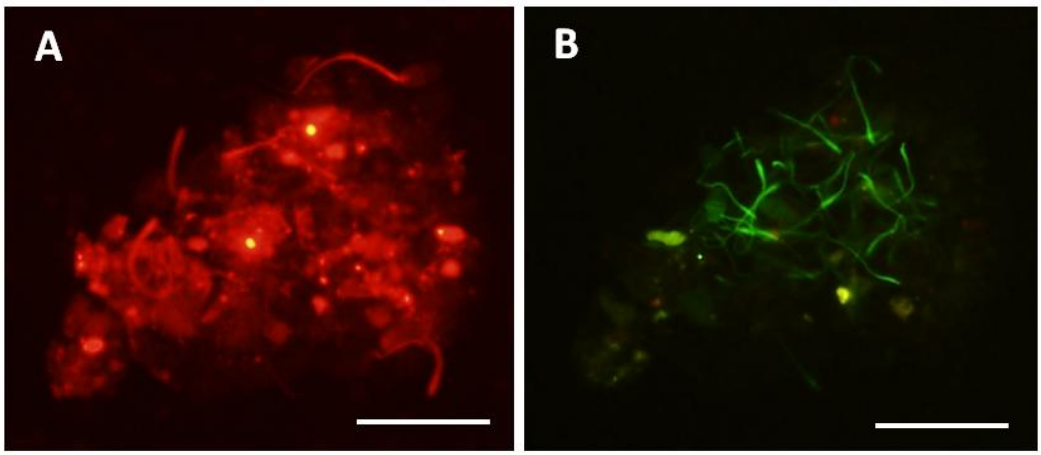

Figure 6 Photomicrographs of CT-Red (A) and CT-Green labeled spermatozoa (B) in the same field which were recovered from left uterine horn flushing.

Scale bars represent 50 µm.

Table 16 Mean±SD of the numbers of recovered spermatozoa from different parts of the reproductive tracts. The sperm recoveries were calculated from the numbers of spermatozoa found in the flushing medium and uterine/oviductal contents.

Method	Segment	Total sperm recovery			
		Left side (CT-Green)		Right side (CT-Red)	
		CT-Green	CT-Red	CT-Green	CT-Red
Flushing	Uterine horn	3,511±3,526.7	505±365.3	1,268±964	4,459±5,189.6
	isthmus	346±473.17	6±4.51	42±50.22	276±305.3
	middle	58±110.8	1±1.73	37±71.84	1±1.15
	ampulla	37±72.17	2±2.08	4±4.35	3±2.65
Oviductal content	isthmus	35.5±66.42	0.67±0.58	0.25±0.5	4±6.93
	middle	0	0	0	0
	ampulla	0	0	0	0

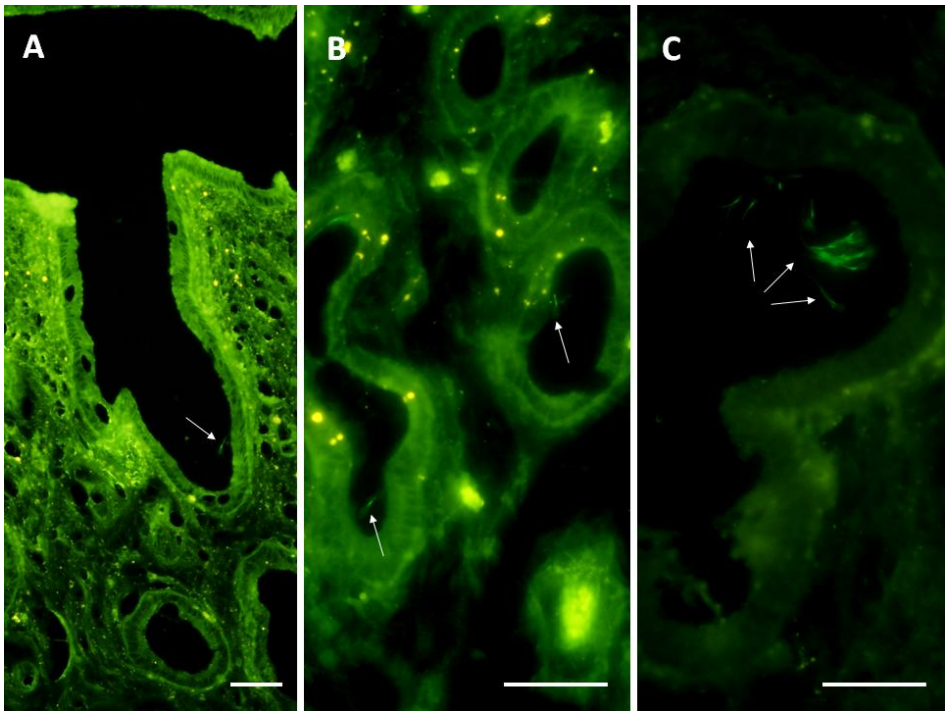

Figure 7 CT-Green labeled spermatozoa (arrow) presented in the uterine lumen (A) and crypt of uterine horn individually (B) or in group (C). Scale bars represent 50 μm.

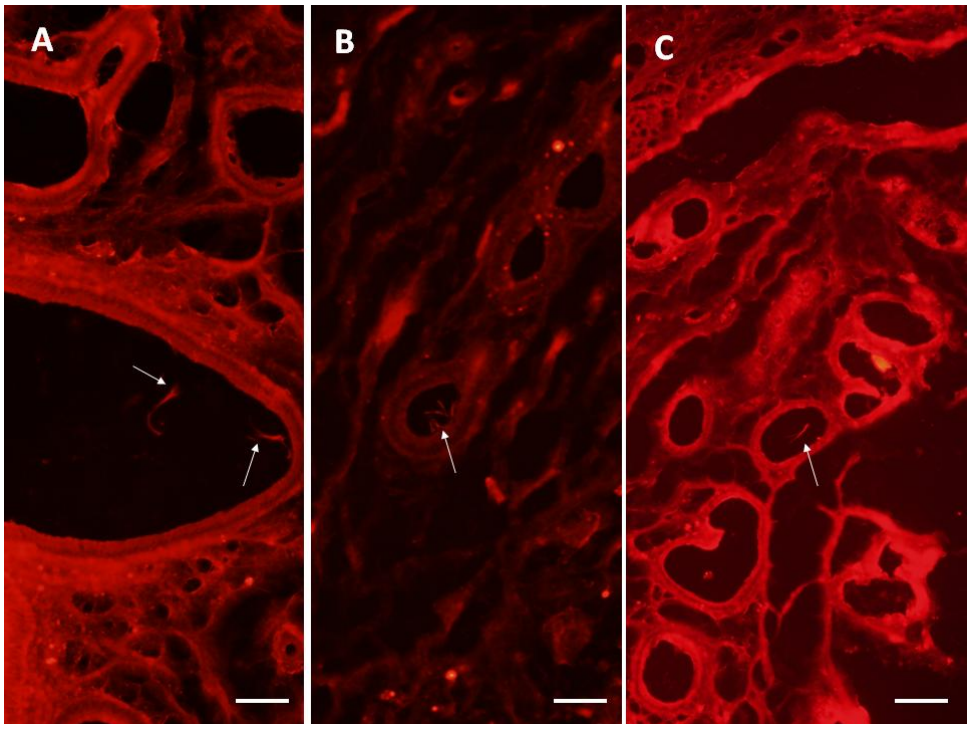

Figure 8 CT-Red labeled spermatozoa (arrow) presented in the uterine lumen (A) and crypt of uterine horn in group (B) or individually (C). Scale bars represent 50 μm.

6.4.3 Experiment 3: the effects of sperm numbers and insemination techniques on pregnancy rate

There was no significant difference in pregnancy rates between any techniques (Table 17). The pregnancy rates were slightly higher when 120 x10^6 spermatozoa were used for LAI. However, this pregnancy rates did not significantly differ between the two sperm doses (P>0.05). Furthermore, pregnancy rates were also not significantly different between single- and double-horn inseminations. For unilateral LAI, the semen deposition into the uterus on ipsilateral side to the observed graafian follicle/ovulation did not significantly improve the pregnancy rates compared with randomly laparoscopic insemination (P>0.05).

Table 17 Pregnancy rates of does inseminated with different numbers of spermatozoa and deposition sites

Insemination dose (x10^6 spermatozoa)	Group	n	Pregnancy (%)			
			Observation*	Non-observation**	Total	
60	1	30	46.67	53.33	50	53.33
	2	30	53.33	60	56.67	
120	3	30	53.33	60	56.67	56.67
	4	30	60	53.33	56.67	

* The does were inseminated ipsilaterally to the observed graafian follicle/ovulation.

** The does were blindly inseminated without ovarian examination.

6.5 Discussion

This study examined the distribution and fertilizing ability of frozen-thawed spermatozoa within in the reproductive tracts of inseminated goats and revealed that goat spermatozoa transited throughout the reproductive tracts even the spermatozoa were laparoscopically inseminated into only one uterine horn. To date, LAI with frozen-thawed semen has become an essential part of controlled breeding in goats, given that LAI provides valuable opportunities to improve reproductive efficiency and to enhance

the genetic improvement with consistently high pregnancy rates (Ritar et al., 1990; Vallet et al., 1992). Although the LAI technique in goats has been efficiently modified from sheep, the results for the LAI in this species have been variable and contradictory (Maxwell, 1986; Correa et al., 1994; Eppleston et al., 1994; Perkins et al., 1996). To overcome this shortcoming, bilateral or unilateral (with ovarian observation) insemination is practically used (Maxwell, 1986). In goat, intrauterine insemination using frozen-thawed semen is mostly performed by bilateral insemination (Ritar et al., 1987; Goonewardene et al., 1996; Martínez-Rojero et al., 2007). However, this technique is time consuming and an extensive manipulation of the reproductive tracts seems likely to elevate prostaglandin F2α levels (Scenna et al., 2005). It would therefore be more efficient if LAI is performed by unilaterally intrauterine insemination, while the pregnancy rate remains similar to that of bilaterally LAI. To study whether spermatozoa deposited into only one uterine horn can be transuterine transport, supravital fluorochromes were used in this study. Fluorochromes have been used to study the sperm distribution, motility, sperm binding and fertilization in several species, including sheep (Druart et al., 2009), rabbit (Parrish and Foote, 1985; 1986), cattle (Miller et al., 1998; Braundmeier et al., 2002; Puglisi et al., 2010), cat (Niu et al., 2006), fowl and turkey hens (King et al., 2002). In this study, the spermatozoa were differently labeled with the CT-Green or CT-Red fluorochromes and examined the sperm quality *in vitro*. Both non-toxic dyes have different emission/excitation wavelengths therefore they cannot be observed at the same time. However, these two fluorochromes were found to be suitable for supravital staining, in terms of sperm viability, motility and also longevity (Table 14). The spermatozoa labeled with these fluorochromes demonstrated high sperm viability and motility (>50%) at 6 h post-labeling (Table 14). The spermatozoa at 6 h after LAI were therefore speculated to be viable, and thus the finding of transuterine transit of the spermatozoa was likely to be the results from the sperm movement. Although we cannot rule out the sperm migration by uterine contractility (Scott, 2000; Langendjik et al., 2002), the presence of spermatozoa within the oviducts and pregnancy rates obtained conclusively indicated that spermatozoa were still viable and motile. This transuterine

movement after unilaterally intrauterine insemination was also found in other species by means of the presence of spermatozoa (Fukushima et al., 2009; Tummaruk et al., 2010) and fertilization (Tsutsui et al., 2000; Tummaruk et al., 2007). Indeed, the distribution of spermatozoa to the contralateral side after single horn insemination was clearly seen in this study but the numbers of recovered spermatozoa were variable among animals, similar to a report in sheep (Quinlivan and Robinson, 1969; Hawk, 1972; Ismaya, 2003). By contrast to the finding that a large numbers of spermatozoa were found in isthmus, UTJ and crypts of uterus, the cumulative numbers of recovered spermatozoa were far less than the total spermatozoa inseminated into the uterus. This suggests that uterine clearance of the inseminated spermatozoa may occur as soon as 6 h post insemination. In addition, spermatozoa observed from cryosections were mostly found in the lumens of the reproductive tracts which was similar to the finding that binding of ram spermatozoa to the surface epithelium was seldom observed (Druart et al., 2009). It was also worth noting that the reproductive tracts of goats were also autofluorescent (Figs. 5, 7 and 8) similar to human cervix (Brookner et al. 2000) and uterine epithelium in sheep (Druart et al., 2010). This autofluoresent intensity has been found to be associated with the metabolism of nicotinamide adenine dinucleotide (NADH) (Brookner et al., 2000).

The results obtained from experiment 3 supported the finding of the experiment 2 indicating that the inseminated spermatozoa could transport to entire reproductive tracts as the pregnancies were obtained after LAI without an observation of the graafian follicle/ovulation. Although pregnancy rate is largely depending on several factors including the amount and motility of ram spermatozoa (Rodriquez et al., 1988; Eppleston et al., 1994), the numbers of spermatozoa used for LAI in goat have been inconsistent and variable from 5 to 200 x 10^6 spermatozoa (Ritar et al., 1990; Baril et al., 1996; Buckrell, 1997; Sohnrey and Holtz, 2005; Martínez-Rojero et al., 2007). Despite of the variations of recommended insemination doses, the pregnancy rates obtained in this study were acceptable (>50%) and similar between the two insemination doses (60 vs. 120x10^6 spermatozoa). Our results also suggested that there was no advantage of ovarian observation at the time of insemination since the experiment 2 and experiment 3

convincingly indicated that the spermatozoa inseminated into the uterine horn could thoroughly transport in the reproductive tracts in terms of the presence of the spermatozoa at the contralaterally deposited site and also the pregnancies were obtained. These results therefore recommend that operation of LAI in goat would be simpler and consume lesser time if the semen is deposited into only one uterine horn without ovarian observation.

In conclusion, frozen-thawed goat spermatozoa can be transuterine migrated following laparoscopic insemination and the total number of 60 $x10^6$ spermatozoa inseminated into one uterine horn without ovarian observation can efficiently be used for laparoscopically intrauterine insemination.

CHAPTER VII

A PRODUCTION OF BLACK GOAT USING LAPAROSCOPIC ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER

7.1 Abstract

Laparoscopic artificial insemination (LAI) and embryo transfer (ET) were used to produce crossbred black-colored goat. In Experiment 1, LAI with frozen-thawed semen of black buck (Australian Melaan) was performed in 75% Saanen crossbred does (white color, n=70). The total numbers of 68 kids were born from 50 does. The skin colors of kids born were black (10.29%), white (39.71%) and other colors (50%). In Experiment 2, two cross-breeding programs were tested including program I: frozen semen of Australian Melaan inseminated to Black Bengal female (n=7) and program II: frozen semen of Black Bengal inseminated to 50% Australian Melaan (n=7). For embryo transfer program, the donors were superovulated and inseminated through laparoscopy with frozen semen at 21 h after estrus. Thirty embryos at 4-8 cell stage (day 3) were surgically collected and transferred into 30 recipients (75% Saanen crossbreed) at approximately 60 h following LAI. Pregnancy rates were 30%. Nine kids born from both programs were black in color with 2.56±0.95 kg birth weight. It is concluded that laparoscopic insemination and embryo transfer can be successfully combined to produce and sustain the genetic potential encoding the black colored skin.

7.2 Introduction

In Thailand, black goats are in demand and more expensive compared to other colors. However, when black goats are mated with goats in other colors, coat colors of offspring are remarkably variable (Asdell and Buchanan Smith, 1928). This is due to the fact that white, brown and tan colors are dominant over black color (Asdell and Buchanan Smith 1928; Adalsteinsson et al., 1994; Sponenberg and LaMarsh, 1996). The skin color is genetically controlled by the presence of two subtypes of melanin pigments (eumelanin and phaeomelanin). The eumelanin is predominantly produced from tyrosine

and represents black color while the other pigment typically expresses reddish brown or tan color. Although melanocytes can produce both types of pigments, eumelanin is genetically recessive (Asdell and Buchanan Smith, 1928). In order to produce black offspring, mating between two breeds of black goats should be performed (Sponenberg et al., 1998). The Australian Melaan (AM) is a black goat breed developed in Australia. This breed is considered hardy, disease resistance and high production. The Black Bengal (BB) is a common name for a small breed black goat found in Bangladesh and Northeast of India. Its skin softness and excellent meat quality are main characteristics (Amin et al., 2000). The genetic improvement of the BB goat could be achieved by selection and cross-breeding (Amin et al., 2000).

Cross-breeding by the application of assisted reproduction technologies (ART) results in the utilization of hybrid vigor for commercial production. Artificial insemination (AI) is a basic tool for genetic improvement worldwide especially with frozen-thawed semen (Leboeuf et al., 2000). In order to get higher pregnancy rates with post-thaw semen, the spermatozoa should be deposited directly into the uterus using laparoscopic artificial insemination (LAI) (Sohnrey and Holtz, 2005). While this crossbred LAI improves the genetics via male germplasm, superovulation followed by embryo recovery and embryo transfer (ET) is a successful method for distributing superior female genes (Isthwar and Memon, 1996). There are two techniques for collecting and transferring embryos, regarding to the stage of embryo development. The embryos beyond eight-cell stage should be collected and transferred to the uterus, while early stage (embryos containing less than 8 cells) should be transferred into the oviducts (Ramon-Ugalde et al., 2008). A higher recovery rate and viability of embryos were reported when embryos were collected in the early stage via oviductal flushing (Ramon-Ugalde et al., 2008). Therefore, this study aimed at examining whether or not the production of black goat offspring using LAI, superovulation as well as ET techniques were efficiently performed.

7.3 Materials and methods

7.3.1 Experimental animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC), Chulalongkorn University (Approval No. 11310030). All female goats, aged between 2 to 4 years old, were used in this experiment. The animal facility was located at the CU-Network for Academic Opportunities and Services, Chulalongkorn University (Nan Province, Thailand, 18°N latitude and 100°E longitude). The animals were fed with concentrates containing 14% (w/w) protein, *ad libitum* of grass with free access to mineral salt blocks and water.

7.3.2 Experimental design

Experiment 1: LAI with frozen-thawed semen of AM was performed in 75% Saanen crossbred does (n=70) in order to study the offspring's coat color.

Experiment 2: Two cross-breeding programs were tested using LAI and ET techniques. These include program I: frozen semen of AM inseminated to BB female (n=7) and program II: frozen semen of BB inseminated to 50% AM (black color does born from experiment 1) (n=7). The donors from both programs were inseminated using LAI technique. The embryos were collected at 60 h after LAI and then transferred to recipients does (75% Saanen crossbred does).

7.3.3 Estrus synchronization

The does in experiment 1 (n=70) were estrus synchronized using intravaginal sponge (Sincro-gest sponges; 65 mg of medroxyprogesterone, Ovejero®, Spain) for 13 days together with an injection of 300 IU pregnant mare serum gonadotropin (PMSG, Folligon®, Intervet Schering-Plough Animal Health, The Netherlands) before sponge removal.

The recipient does in experiment 2 (n=40) were estrus synchronized at the same period as the donors. The program was as described above with an injection of 200 IU human chorionic gonadotropin (hCG; Chorulon®, Intervet Schering-Plough Animal Health, The Netherlands) when the estrus sign was detected in order to induce an ovulation.

7.3.4 Superovulation

The donors in experiment 2 (n=14) were synchronized and superovulated as previously described by Shin et al. (2008) and Lehloenya and Greyling (2010) with minor modifications. The estrous cycle of the donors was controlled by an intravaginal progesterone sponge (Sincro-gest sponges; 65 mg of Medroxyprogesterone, Ovejero®, Spain) for 13 days. A total of 200 mg of follicle stimulating hormone (FSH; Folltropin V®, Bioniche Animal Health, Vetrepharm, Canada) was divided into 7 doses (50, 25, 25, 25, 25, 25 and 25 mg). The FSH was then twice-daily administrated. The first dose was started on day 10^{th} after the progesterone sponge insertion. Two doses of cloprostenol (125 μg/ml, Estrumate®, Schering-Plough Animal Health, NJ, USA) were administered intramuscularly at the time of the sixth and seventh FSH injections. Estrus signs were detected every 6 h using apronized bucks. When the does were in estrus, 200 IU of hCG (Chorulon®, Intervet Schering-Plough Animal Health, The Netherlands) was given to induce ovulation and the LAI was then performed 21 h later.

7.3.5 Laparoscopic artificial insemination (LAI)

All does submitted to the operation were restricted from feed for 24 hours and water for 12 hours (Mobini et al., 2012). They were intravenously anesthetized with xylazine HCl (0.1 mg/kg) and ketamine HCl (4.4 mg/kg). The analgesic drug (0.04 mg/kg phenylbutazone) and antibiotic (20,000 IU/kg penicillin-streptomycin) were also given intramuscular. The does were positioned on a trolley in dorsal recumbency with the head tilted down at about 45° angle. After ovarian observation to confirm the presence of graafian follicles, post-thaw sperm (60×10^6 spermatozoa in 0.25 ml) were inseminated into the lumen of each uterine horn at a halfway between the utero-tubular junction (UTJ) and the bifurcation (Ritar et al., 1990).

7.3.6 Embryo transfer (ET)

Approximately 60 h following AI, the embryos were surgically collected from the donors. The goats were anesthetized as described in LAI. The numbers of recovered embryos were recorded in relation to the number of corpus luteum (CL). The reproductive tract of a donor was accessed through a mid-ventral incision. Embryos at 4-8 cell stage (day 3) were collected from superovulated donors by oviductal flushing

with sterile phosphate-buffered saline infiltrated through the butterfly needle (25G, 19 mm) (Figs. 9A and 9B). The flushing medium was collected via a polyethylene tube with outer diameter of 1.57 mm and inner diameter of 1.14 mm (Intramedic®, Becton, Dickinson and Company, N.J., USA). The flushing fluid was recovered in a sterile plastic Petri dish and the embryos were immediately searched and counted under a stereomicroscope (80x) (Nikon SMZ645, Japan). The recovered embryos were classified according to the gross morphology and only excellent and good embryos were used for ET. The fresh embryos were transferred into the oviducts of recipients ipsilateral to the presence of CL via the insertion of an IVF catheter (Veterinary concepts®, Australia) into the oviduct (Fig. 9C).

7.3.7 Pregnancy diagnosis

The pregnancy was confirmed at 45 days after LAI or ET by real time B-mode ultrasonography (HS-2000, Honda Electronics Co., Ltd., Japan). The skin color and birth weight of kids were recorded.

7.3.8 Statistical analysis

The data were descriptively analyzed. The percentage of goat showing estrus sign, pregnancy and embryo recovery are reported. The birth weights of kids are presented as mean±SD. The frequency of kids in each color was expressed as a percentage of the total number of kids born.

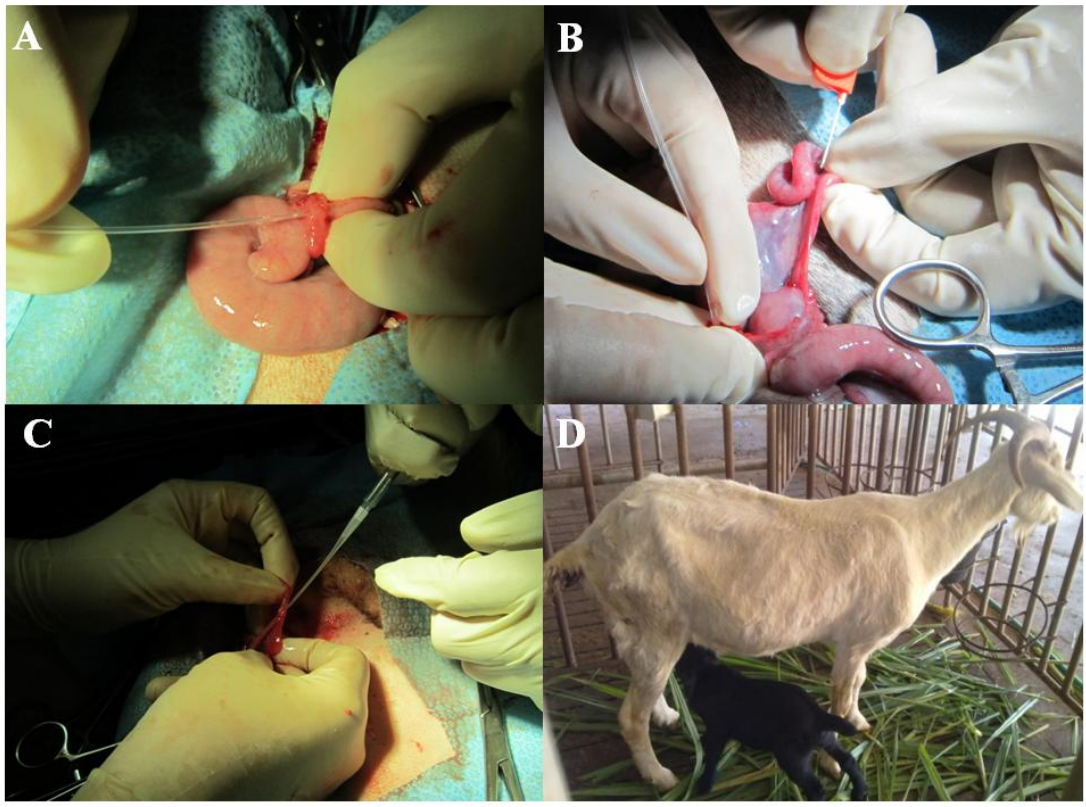

Figure 9 Oviductal flushing in superovulated donors. A: insertion of the polyethylene tube via fimbria and infundibulum and holding with fingers. B: infiltration the flushing medium at utero-tubular junction using a butterfly needle. C: insertion of an IVF catheter into the oviduct via the infundibulum of recipient to transfer the embryo. D: black kid was born by transfer the embryo to crossbred Saanen.

7.4 Results

Experiment 1: A total numbers of 68 kids were born from 50 does (pregnancy rates=71.43%). The amounts of kids with black colored skin were lower than other colors (Table 18). The 35 does (70%) delivered one kid while other 15 does gave birth to twins and triplets (12 and 3 does, respectively). The differences in colors were observed among twin and triplet. The average of birth weight was 2.87±0.61 kg.

Table 18 The skin colors of goat offspring from Australian Melaan (frozen semen) and 75% Saanen crossbred does

Offspring	Skin color			Total
	Black	White	Other	
n	7	27	34	68
%	10.29	39.71	50	

Experiment 2: All donors and recipients does (35 from 40, 87.50%) came into estrus after synchronization. The mean number of CL (ovulation) in donor was 13±6.6 (ranging from 8 to 32) while the recovery rate (unfertilized oocytes and embryos) varied from 0 to 100% (55.85±35.69%). The transferable embryos, at 2-8 cell stage when embryo collection, were performed on day 3 after LAI. The number of embryos collected from two breeding programs (Table 19). Thirty good embryos from 11 donors (BB=7, 50%AM=4; 0% recovery rate in three donors) were transferred into 30 recipient does. The pregnancy rates were 30% (9/30). All nine kids were healthy and black in color (Fig. 9D) with 2.56±0.95 kg birth weight.

Table 19 The number of embryos collected from two breeding programs

Breeding program	Frozen semen	Donor	No. of donors	No. of embryos	No. of kids
1	AM	BB	7/7	18	6
2	BB	50%AM	4/7*	12	3

AM = Australian Melaan, BB = Black Bengal

*Recovery rate = 0 in three donors

7.5 Discussion

We found that the LAI and ET were successfully performed to maintain the phenotypic expression of black offspring when black goats were mated together. Therefore, when frozen semen of AM was inseminated to white Saanen does, the lower

percentages of black kids were born as shown in experiment 1. This is due to the recessive eumelanin which controls black color, mating between black and black goats produces black offspring whereas mating between black goats and goats of other colors results in variable coat colors (Asdell and Smith, 1926; Adalsteinsson et al., 1994; Sponenberg et al., 1998).

In an embryo collection, the wide range of the success in recovery rate was in agreement with a previous study indicating that the results can vary from complete failure to extremely high success (Baldassarre and Karatzas, 2004). It was noted that a high recovery rates were mostly obtained in does that had never been received hormonal treatment. Moreover, we found that repeated treatments especially for PMSG and FSH (porcine origin) negatively affected on the ovarian responses and also embryo recovery rate as remarked by Remy et al. (1991) and Cognie (1999). In addition, internal organ adhesion from previous LAI and surgical ET also impair the procedure during embryo collection (Suyadi et al., 2000).

All black offspring born from both breeding programs were healthy with higher birth weight (2.56±0.95) than the average birth weight of BB kids born at this breeding center (1.81±0.26 kg) or in Bangladesh (1.08±0.06 kg, Paul et al., 2011) but slightly lower than kids born in experiment 1 (2.87±0.61). This black colored skin is controlled by the production of melanin pigments from melanocytes in response to the *alpha* melanocyte stimulating hormone (Jackson, 1994; Hoekstra, 2006). Although melanocytes can produce both eumelanins and phaeomelanins, the phenotypic expression of the black colored hair and skin is mediated by only eumelanin. While the mechanism that controls the hair and skin color is well described in mouse, similar mechanisms remain to be studied in goat. Practically, our study demonstrated that crossbred black goats can be produced via LAI and ET. The production of these crossbred goats would be phenotypically and genetically beneficial means of climate adaptation, disease tolerant and also meat production. LAI and ET combined technique provides a valuable practical opportunity to improve reproductive efficiency and to enhance the genetic improvements.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSION

8.1 General discussion: Semen cryopreservation, Laparoscopic artificial insemination and embryo transfer in goat in Thailand

Generally, goat sperm are sensitive to cryopreservation as sperm quality and pregnancy rate are usually poorer than that obtained from non-cryopreserved sperm. It has become clear that suboptimal freezing induces irreversible cellular damages in particular at the levels of sperm plasma membrane (Ortman and Rodriguez-Martinez, 1994), acrosomal membrane (Jones and Martin, 1973) and sperm mitochondria (Watson, 1995), etc. As a result, pregnancy rates after transcervical insemination in goat remain poor compared to the direct deposition of frozen-thawed semen to the uterine horn via a laparoscopic insemination (Sohnrey and Holtz, 2005). For this reason, improving techniques aimed specifically at cryopreserving goat sperm without significant loss of sperm quality are sought to be important for goat breeding industry. Among factors affecting the freezability of spermatozoa, supplementation of freezing medium with various types of cryoprotectant plays an important role in minimizing the physical and chemical stresses occurred during cryopreservation procedure (Purdy, 2006; Peterson et al., 2007). Although it is generally accepted that high concentration of cryoprotectant reduces lethal intracellular ice formation (Mazur and Kleinhans, 2008), the toxicity of the particular cryoprotectant is frequently found (Fahy, 2010).

Glycerol is the most widely used as a membrane-permeable cryoprotectant for preserving mammalian spermatozoa (Ritar and Salamon, 1982; Tuli and Holtz, 1994). It is superior for protecting buck spermatozoa during cryopreservation to other chemicals (Leboeuf et al., 2000; Purdy, 2006; Peterson et al., 2007). However, suitable amount of glycerol added into the freezing medium remains controversial. The amounts of glycerol being used are likely to differ between the breeds and individual males (Holt, 2000[b]). The excess amount of glycerol induces osmotic damage to spermatozoa, hence reducing post-thaw semen quality (Rudenko et al., 1984; Holt, 2000[a]). On the other

hand, too low glycerol concentration poorly protects the spermatozoa against cryoinjury (Mazur, 1984). The range of 3 to 14% glycerol are generally used in freezing extender for several goat breeds (Waide et al., 1977; Leboeuf et al., 2000) while 5 and 7% glycerol are frequently reported for Black Bengal breed (Biswas et al., 2002; Afroz et al., 2008; Apu et al., 2012), but we found that the 7% glycerol protocol poorly protected the spermatozoa against cryoinjury during freezing and thawing procedure. The different results may due to the different freezing extender compositions, and also the different method of evaluation. On the other hand, poor sperm quality obtained from a high glycerol concentration probably causes by an extreme osmotic stress that can damage the cell structures and impair the sperm motility (Deka and Rao, 1986; Gil et al., 2003; Sönmez and Demirci, 2004; Sundararaman and Edwin, 2008). The appropriate amount of glycerol increases the membrane fluidity by rearranging the membrane lipid and protein, thus increasing the survival ability of spermatozoa during cryopreservation (Holt, 2000[a]).

The surfactants either sodium dodecyl sulfate (SDS) (Aboagla and Terada, 2004[a,b]) or Equex STM paste have also been reported to affect the freezing ability. For goat semen, beneficial effects of trehalose-egg yolk extender containing SDS were reported on acrosome integrity and motility (Aboagla and Terada, 2004[a,b]). Equex STM Paste gives similar result to SDS by improving not only acrosome integrity and motility but also viability and normal tail spermatozoa. It improves the post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell to protect spermatozoa against the toxic effects of glycerol during the freeze-thaw processes (Martin et al., 1979; Arriola and Foote, 1987). In the present study, the percentage of intact acrosome spermatozoa was higher in Equex STM Paste supplemented group. The cryoprotective effect of Equex STM Paste on the acrosomal membrane is crucial since the presence of intact acrosome is necessary during the fertilization process and has been reported to highly correlate with fertility of frozen-thawed semen (Saacke and White, 1972). However, the beneficial effect of Equex STM Paste on intact acrosome was only observed when the egg yolk was also present. The Equex STM Paste is therefore believed to act via the

modifications of egg yolk lipoproteins (Peña and Linde-Forsberg, 2000; Arriola and Foote, 1987). However, it seems likely that prolonged incubation of sperm in freezing extender supplemented with Equex STM Paste has a negative effect on sperm membrane/viability probably by the toxic effect of Equex STM Paste (Axnér et al., 2004). From the results in chapter II, Equex STM Paste enhanced the ability of spermatozoa to sustain in the incubation at temperatures close to the female body temperature for 2 h. This finding confirms previous observations on successful semen freezing using freezing extender containing Equex STM Paste in many species (Holt, 2000[a]). However, the beneficial effects were in a dose dependent manner. Therefore, in chapter IV, we examined the effects of freezing extender containing different concentrations of Equex STM Paste and found that the optimal concentration of Equex STM Paste for goat semen freezing was 0.50% (v/v) in glycerol-egg yolk based freezing extender. An increasing of Equex STM Paste concentrations more than 0.75% resulted in a sharp reduction in the velocity parameters and had no benefit to the plasma membrane integrity as compared with the lower concentrations. The beneficial effects were observed at immediately after thawing and during 3 h incubation after thawing. We did not observe detrimental effects of Equex STM Paste on acrosomal membrane integrity irrespective of the amount of Equex STM Paste supplemented. This result was different from previous reports in that too high concentrations of SDS (active component of Equex STM Paste) affected to the acrosomal membrane integrity (Dewit et al., 2000; El-Kon et al., 2010).

Egg yolk also works synergistically with surfactants such as Equex STM Paste and its active compound sodium dodecyl sulphate (SDS) (Peterson et al., 2007). Although the mechanisms of Equex STM Paste and SDS in protecting spermatozoa are not completely known, they have been demonstrated to reduce the toxic effects of glycerol and to stabilize acrosomal membrane during cooling and thawing (Jimenez, 1987). However, it is worth nothing that the benefits of Equex STM Paste are more pronounced when egg yolk is added into the freezing extender (Pursel et al., 1978; Dewit et al., 2000; Aboagla and Terada, 2004[a]). This is likely because Equex STM Paste acts as an emulsifier of the egg yolk lipids, thereby making it more accessible to the

sperm membrane during cryopreservation (Amann and Pickett, 1987; Buhr and Pettitt, 1996; Dewit et al., 2000).

In order to get an achievement of AI program, semen should be collected in an appropriate time with a proper management of semen storage and use (Leboeuf et al., 2000). We conducted the chapter 5 to determine the quality and freezability of semen collected from crossbred Saanen born and raised in humid and tropical condition at different seasons of the year. These crossbred Saanen still show seasonal variation on semen production and freezability, although many studies reported about a seasonal breeder in goat raised in higher latitudes (higher than 35˚N) during short day period (Corteel, 1977; Leboeuf et al., 2000; Barkawi et al., 2005) (Chemineau et al., 1987; Janett et al., 2003). Our finding coincided with the findings of Ahmed et al. (1997) who established the seasonal change on reproductive behavior in Sudan (15°30´N, 32°E). Despite of a little variation in day length in this area (18°N, 100°E), the temperature and humidity were hypothesized to play an important role on these effects. Overall, the fresh semen collected in summer and winter provided a higher in quality than in rainy season. The information about semen quality collected in rainy season is variable in other studies. A higher semen quality in rainy was reported due to higher rainy season feed (Carmenate and Gamcik, 1982) that provided positive influence on nutrition in goat (Zarazaga et al., 2009) and ram (Martin and Walkden-Brown, 1995). However, this effect did not exist in our experiment which in agreement with another report in sheep (Gordon, 1997). From our results, a lot of semen parameters, except for volume, total sperm per ejaculate and motility were significant lower in rainy. The highest in maximum relative humidity together with a high degree of mean temperature in rainy season resulted in an increasing of THI, which was explained to be a cause of heat stress and affected a sperm production (Mukherjee et al., 1953; King, 1993).

For frozen semen, the post-thaw semen qualities were not significant different except for motility characteristics and plasma membrane integrity which showing their best in winter, although the characteristic of fresh semen collected in winter was not dramatically superior to other seasons. However, the frozen semen quality was

acceptable for AI using during all seasons of the year. In conclusion, despite of the difference among season of the spermatozoa, the quality of fresh semen throughout the year provided accepted quality after thawing in every season. However, the best motility characteristics and sperm with intact plasma membrane were shown in winter.

Despite of the appropriate time and technique for collecting semen as well as an improvement in freezing and thawing procedure of frozen-thawed goat semen, some population of spermatozoa are damaged. Therefore, LAI which semen is deposited closer to the site of fertilization provides a higher pregnancy rate than that of cervical AI in goat (Epstein and Herz, 1964; Ritar et al., 1990[a]; Sohnrey and Holtz, 2005). However, most LAI protocols in goat obtained from sheep, which is still conflicting about higher pregnancy rate in unilateral or bilateral insemination (Maxwell, 1986; Correa et al., 1994; Eppleston et al., 1994; Perkins et al., 1996). Moreover, some impairment may occur in LAI if too small number of spermatozoa is deposited (Hunter et al., 1982). The successful rates depend largely on the amount and motility of ram spermatozoa (Rodriquez et al., 1988; Eppleston et al., 1994). The study in chapter VI comprising stepwise methods to exam the sperm trait in female genital tract of goat, which prove that inseminated with $60x10^6$ post-thaw spermatozoa in only one horn without regard of ovulation side was sufficient for LAI wuth frozen semen in goat. Therefore, the operation of LAI in goat would be simpler and consume lesser time because semen deposition in only one uterine horn without ovarian examination was sufficient.

In chapter VII, the cross-breeding program was used to utilize of hybrid vigor for commercial production. In Thailand, black goats are in demand and more expensive compared to other colors. In order to produce black offspring, mating between two breeds of black goats must be performed since eumelanin which represents black color is genetically recessive. Practically, our study demonstrated that LAI and ET were successfully performed to maintain the phenotypic expression of nine black kids born. These techniques have become an essential and integral part of controlled breeding in goats and provided valuable practical opportunities to improve reproduction efficiency

and to enhance the genetic improvement. It provides a superior and more consistent pregnancy rates.

8.2 Further direction and industrial application

Due to the trade internationally, the distribution of genetic is rapidly increase. The future investigation in regard to the application of reproductive technologies for genetic improvement will be good choices for increasing production of goat in both meat and dairy. The goat industries in Thailand are gradually increasing too. However, the research in goat production in Thailand has been limited compared with other species. It is imperative that research and training in goat production should be given more attention, if goat development is to be realized. The research for LAI and ET which lack of the risk of transmitting infectious disease via semen cryopreservation and frozen embryo will be advantageous for goat industry. The freezing protocol should be improved in order to increase the longevity of spermatozoa such as using a control rate freezer which provides more reliable result in temperature control. The techniques in transcervical AI which is more practical in farm animal should me developed for high conception rate. Moreover, the study for practical hormone regimens for superovulation and estrus synchronization can increase the success rate in embryo transfer.

8.3 Conclusion

The semen cryopreservation, LAI and ET are the three techniques of assisted reproductive technologies (ART) that we studied in this research and aimed to future applied in goat industry. An improvement of semen freezing via semen extender supplementation and a study on seasonal effect on semen production and quality were investigated. We also showed that the goat sperm can distribute to the opposite side after unilateral insemination, this makes more rapid and practical to do LAI in goat and result in a genetic improvement with an acceptable pregnancy rate. Moreover, the practical techniques for LAI and ET were applied to the goat industry for producing goat offspring which are in demend of consumers.

REFERENCES

Aboagla, E.M.-E. and Terada T. 2003. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol Reprod. 69:1245-1250.

Aboagla, E.M.-E. and Terada, T. 2004[a]. Effects of the supplementation of trehalose extender containing egg yolk with sodium dodecyl sulfate on the freezability of goat spermatozoa. Theriogenology 62: 809-818.

Aboagla, E.M.-E. and Terada, T. 2004[b]. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology 62: 1160-1172.

Adalsteinsson, S., Sponenberg, D.P., Alexieva, S. and Russel, A.J.F. 1994. Inheritance of goat coat colors. J Hered. 85: 267-272.

Afroz, S., Islam, Md.R., Khandoker, M.A.M.Y. and Akter, Q.S. 2008. Cryopreservation of Black Bengal buck semen: Effects of diluents and freezing on sperm motility and morphology. Anim Sci J. 79: 550-553.

Ahmad, N. and Noakes, D.E. 1995. Seasonal variations in testis size, libido and plasma testosterone concentrations in British goats. Anim Sci. 61: 553–559.

Ahmed, M.M.M., Makawi, S.A. and Gadir, A.A. 1997. Reproductive performance of Saanen bucks under tropical climate. Small Rumin Res. 26: 151-155.

Aitken, R.J., Sutton, M., Warner, P. and Richarson, D.W. 1985. Relationship between the movements characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona free hamster oocytes. J Reprod Fertil. 73(2): 441-449.

Akourki, A., Gil., L., Echegaray, A., Espinosa, E., Josa., A., De Blas, I., Gonzalez, N., Gallegos de la Hoya, M., and Meque, L.C. 2004. Effect of the extender supplement equex-stm on cryopreserved semen in the Assaf sheep. Cryoletters 25(2): 147-154.

Al-Ghalban, A.M., Tabbaa, M.J. and Kridli, R.T. 2004. Factors affecting semen characteristics and scrotal circumference in Damascus bucks (technical note). Small Rumin Res. 53: 141–149.

Amann, R. and Pickett, .B. 1987. Principles of cryopreservation and a review of stallion spermatozoa. J Equine Vet Sci. 7: 145-173.

Amaro, S.F., Lemoine, R.J. and Trejo, G.A. 1997. Efecto de la dosis del progestágeno y del tipo de diluent sobre la fertilidad en cabras criollas inducidas a estro 60 días posparto e inseminadas con some congelato. Memorias de la XII Reunión Nacional Sobre aprinocultura. AMPA. Torreón, Coal, México: 29-34.

Amin, M.R., Husain, S.S. and Islam, A.B.M.M. 2000. Evaluation of Black Bengal goats and their cross with the Jamunapari breed for carcass characteristics. Small Rumin Res. 38: 211-215.

Amundson, J.L., Mader, T.L., Rasby, R.J. and Hu, Q.S. 2006. Environmental effects on pregnancy rate in beef cattle. J Anim Sci. 84: 3415-3420.

Anakkul, N., Suwimonteerabutr, J., Singlor, J., Phutikanit, N., Tharasanit, T. and Techakumphu, M. 2011. Effect of Equex STM Paste on the quality and motility characteristics of post thawed cryopreserved goat semen. Thai J Vet Med. 41: 345-351.

Apu, A.S., Husain, S.S., Khandoker, M.A.M.Y., Rahman, A.H.M.S. and Notter, D.R. 2008. Investigation on seminal attributes and fertility of Black Bengal goat. Bang J Anim Sci. 37: 17-24.

Apu, A.S., Khandoker, M.A.M.Y., Husain, S.S., Fakruzzaman, M. and Notter, D.R. 2012. A comparative study of fresh and frozen-thawed semen quality in relation to fertility of Black Bengal goats. Iranian J App Anim Sci. 2: 157-161.

Armstrong, D.V. 1994. Heat stress interaction with shade and cooling. J Dairy Sci. 77: 2044-2050.

Armstrong, D.T. and Evans, G. 1983. Factors influencing success of embryo transfer in goats. Theriogenology 19: 31-42.

Armstrong, D.T., Pfitzner, A.P., Wames, G.M. and Seamark, R.F. 1983. Superovulation treatments and embryo transfer in Angora goats. J Reprod Fertil. 67: 403-410.

Arriola, J. and Foote, R.H. 1987. Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. J Dairy Sci. 70: 1664–1670.

Asdell, S.A. and Smith, A.D.B. 1926. Inheritance of color, beard, tassels and horn in the goat. J Hered. 425-430.

Asher, G.W., Peterson, A.J. and Bass, J.J. 1989. Seasonal pattern of LH and testosterone secretion in adult male fallow deer. *Dama dama*. J Reprod Fert. 85: 657-665.

Averill, R.L.W. and Rowson, L.E.A. 1958. Ovum transfer in sheep. J Endocrinol.16: 326-336.

Axnér, E., Hermansson, U. and Linde-Forsberg, C. 2004. The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim Reprod Sci. 84: 179–191.

Azerêdo, G.A., Esper, C.R. and Resende, K.T. 2001. Evaluation of plasma membrane integrity of frozen-thawed goat spermatozoa with or without seminal plasma. Small Rumin Res. 41: 257–263.

Baldassarre, H. and Karatzas, C.N. 2004. Advanced assisted reproduction technologies (ART) in goats. Anim Reprod Sci. 82–83: 255–266.

Baril, G., Pougnard, J.L., Freitas, V.J.F., Leboeuf, B. and Saumande, J. 1996. A new method for controlling the precise time of occurrence of the preovulatory gonadotropin surge in superovulated goats. Theriogenology 45: 697-706.

Barkawi, A.H., Elsayed, E.H., Ashour, G. and Shehata, E. 2006. Seasonal changes in semen characteristics, hormonal profiles and testicular activity in Zaraibi goats. Small Rumin Res. 66: 209–213.

Bilodeau, J.F., Blanchette, S., Cormier, N. and Sirard, M.A. 2002. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. Theriogenology 57: 1105–1122.

Biswas, D., Bari, F.Y., Shamsuddin, M., Rahman, M.M. and Rahman, M.M. 2002. Determination of glycerol percentages for preserving the Black Bengal buck (*Capra hircus*) spermatozoa for long time. Pak J Biol Sci. 5: 715-718.

Bittencourt, R.F., Filho, A.L.R., Lima, M.C.C., Alves, S.G.G., Vasconcelos, M.F., Biscarde, C.E., Leal, L.S. and Oba, E. 2008. Effect of a calcium chelator, a detergent and the soybean lecithin on the quality of the frozen-thawed goat semen. Braz J Vet Res Anim Sci. 45: 305-312.

Bollwein, H., Fuchs, I. and Koess, C. 2008. Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. Reprod Dom Anim. 43: 189–195.

Braundmeier, A.G., Demers, J.M., Shanks, R.D., Saacke, R.G. and Miller, D.J. 2002. Examination of the binding ability of bovine spermatozoa to the zona pellucida as an indicator of fertility. J Androl. 23(5): 645-651.

Brookner, C.K., Follen, M., Boiko, I., Galvan, J., Thomsen, S., Malpica, A., Suzuki, S., Lotan, R. and Richards-Kortum, R. 2000 Autofluorescence patterns in short-term cultures of normal cervical tissue. J Photoch Photobio B. 71: 730–736.

Bucak, M.N., Sarıözkan, S., Tuncer, P.B., Sakin, F., Ateşşahin, A., Kulaksiz, R. and Çevik, M. 2010. The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Rumin Res. 89: 24-30.

Buckrell, B. 1997. Reproductive technologies in commercial use for sheep, goats, and farmed deer. Proceedings of the Society for Theriogenology, Nashville, TN. 185.

Budworth, P.R., Amann, R.P. and Hammerstedt, R.H. 1987. A microcomputer-photographic method for evaluation of motility and velocity of bull sperm. J Dairy Sci. 70: 1927-1936.

Buhr, M.M. and Pettitt, M.J. 1996. Frozen-thawed boar sperm: isolation of membranes and fluidity measurement. Reprod Dom Anim. 31: 147-152.

Buranaamnuay, K., Tummaruk, P., Singlor, J., Rodriguez-Martinez, H. and Techakumphu, M. 2009. Effects of straw volume and Equex-STM® on boar sperm quality after cryopreservation. Reprod Dom Anim. 44:69-73.

Carmenate, P.C. and Gamcik, P. 1982. Physical and morphological properties of ram semen in different seasons. Folia Veterinaria Bratislava. 26: 39-52.

Cameron, R.D.A. and Blackshaw, A.W. 1980. The effect of elevated ambient temperature on spermatogenesis in the boar. J Reprod Fert. 59: 173-179.

Catunda, A.G.V., Aguiar, G.V., Lima, Í.C. S., Pereira, J.F., Pitombeira, R.S.S., Andrade, I.R.A., Martins, G.A., Souza, C.E.A., Nunes, J.F., Moura, A.A.A.N. and Campos, A.C.N. 2011. Characterization of ejaculates from crossbreed male goats raised in tropical environment. Rev Port Cienc Vet. 110: 59-67.

Chang, Y.C., Jung, G.K., Sang, R.C., Dong, S.S., Young, K.K., Balasubramanian, S., Sang, Y.C. and Gyu, J.R. 2006. Influence of seasons, extenders, slow and rapid rreezing on seminal characters in Korean native bucks. Reprod Dom Anim. 41: 55–60.

Chemineau, P., Ba, G., Leboeuf, B., Maurel, M.C., Roy, F., Pellicer-Rubio, M., Malpaux, B. and Cogníe, Y. 1999. Implications of recent advances in reproductive physiology for reproductive management of goats. J Reprod Fertil. 54: 129–142.

Chemineau, P. and Cognié, Y., 1991. Training manual on artificial insemination in sheep and goats. FAO, Rome, Italy. 222pp.

Chemineau, P., Daveau, A., Maurice, F. and Delgadillo, J. 1987. Effects of tropical photoperiod on sexual activity of Alpine goats (Abstract). Proc. 4th Int. Conf. on Goats, Brasília, Brazil. 269.

Chemineau, P. and Xande, A., 1982. Reproductive efficiency of Creole meat goats permanently kept with males. Relationship to a tropical environment. Trop Anim Prod. 7: 98–104.

Cheng, F.P., Wu, J.T., Chan, J.P.W., Wang, J.S., Fung, H.P. and Colenbrander, B. 2004. The effect of different extenders on post-thaw sperm survival, acrosomal integrity and longevity on cryopreserved semen of Formosan Sika deer and Formosan Sambar deer. Theriogenology 61: 1605–1616.

Choe, C.Y., Kim, J.G., Cho, S.R., Son, D.S., Kim, Y.K., Balasubramanian, S., Choe, S.Y. and Rho, G.J. 2006. Influence of seasons, extenders, slow and rapid freezing on seminal characters in Korean native bucks. Reprod Dom Anim. 41: 55-60.

Churg, A., Zaneveld, J.D. and Schumacher, F.B. 1973. Detergent treatment of human and rabbit spermatozoa: Ultrastructural changes and release of midpiece enzymes. Biol Reprod. 10: 429-437.

Cognie, Y. 1999. State of the ART in sheep-goat embryo transfer. Theriogenology 51: 105-116.

Cognié, Y., Baril, G., Poulin, N., Mermillod, P. 2003. Current status of embryo technologies in sheep and goat. Theriogenology 59, 171-188.

Correa, J., Bergmann, B. and Gatica, R. 1994. Fertilization rate in sheep unilaterally inseminated with frozen semen. Small Rumin Res. 13: 99-101.

Corteel, J.M. 1974. Viability of goat spermatozoa deep frozen with or without seminal plasma: glucose effect. Ann Biol Anim Biochim Biophys. 14(4-B): 741–745.

Corteel, J.M., 1977. Production, storage and artificial insemination of goat semen. In: Management of Reproduction in Sheep and Goats Symposium. Madison, July 24–25, 1977. 41–57.

Corteel, J.M., 1981. Collection, proceeding and artificial insemination of goat semen. In: Goat Production. C. Gall (ed.). London: Academic Press. 171-191.

Corteel, J.M., Baril, G. and Leboeuf, B. 1980. Residual seasonal variations in fertility in selected deep-frozen ejaculates of European dairy male goats. In: 9th Int. Congr. Anim. Reprod. A.I. Madrid. 5: 422–425.

Corteel, J.M., Leboeuf, B. and Baril, G. 1988. Artificial breeding of adult goats and kids induced with hormones to ovulate outside the breeding season. Small Rumin Res. 1: 19–33.

Cupps, P.T., Mcgowan, B., Rahlmann, D.F., Reddon, A.R. and Weir, W.C. 1960. Seasonal changes in the semen of rams. J Anim Sci. 19: 208-213.

Daudu, C.S. 1984. Spermatozoa output, testicular sperm reserve and epididymal storage capacity of the Red Sokoto goats indigenous to northern Nigeria. Theriogenology 21: 317–324.

Deka, B.C. and Rao, A.R. 1986. Effect of glycerol level in Tris-based extender and equilibration period on quality of frozen goat semen. Theriogenology 26: 231-238.

Deka, B.B. and Rao, A.R. 1987. Effect of extenders and thawing methods on post-thawing preservation of goat semen. Ind Vet J. 64: 591–594.

Dewit, M., Marley, W.S., and Graham, J.K. 2000. Fertilizing potential of mouse spermatozoa cryopreserved in a medium containing whole eggs. Cryobiology 40: 36-45.

Delgadillo, J.A., Leboeuf, B. and Chemineau, P. 1991. Decrease in the seasonality of sexual Behavior and sperm production in bucks by exposure to short photoperiodic cycles. Theriogenology 36: 755–770.

Delgadillo, J.A., Malpaux, B. and Chemineau, P. 1997. Reproduction of goats in the tropics and subtropics. Prod Anim. 10:33-41.

Donnelly, E.T., Lewis, E.M.S., McNally, J.A. and Thompson, W. 1998. In vitro fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. Fertil Steril. 70(2): 305-314.

Dorado, J., Hidalgo, M., Muñoz, A. and Rodríguez, I. 2009. Short communication: Assessment of goat semen freezability according to the spermatozoa characteristics from fresh and frozen samples. Anim Repro Sci. 112(1-2): 150-157.

Druart, X., Cognié, J., Baril, G., Clément, F., Dacheux, J.L. and Gatti, J.L. 2009. *In vivo* imaging of in situ motility of fresh and liquid stored ram spermatozoa in the ewe genital tract. Reproduction 138: 45–53.

Ehling, C., Wirth, P., Schindler, L., Hadeler, K.G., Döpke, H.H., Lemme, E., Herrmann, D. and Niemann, H. 2003. Laparoscopical intrauterine insemination with different doses of fresh, conserved, and frozen semen for the production of ovine zygotes. Theriogenology 60: 777–787.

El-Kon, I.I., Sallam, A.A., Ashmawy, T.M. and Heleil, B. 2010. Effect of sodium dodecyl sulfate (SDS) on the viability and fertility of Damascous goat spermatozoa. Global Veterinaria 4: 576-581.

Eppleston, J. and Maxwell, W.M.C. 1995. Sources of variation in the reproductive performance of ewes inseminated with frozen-thawed ram semen by laparoscopy. Theriogenology 43: 777.

Eppleston, J., Salamon, S., Moore, N. and Evans, G. 1994. The depth of cervical insemination and site of intra-uterine insemination and their relationship to the fertility of frozen thawed ram semen. Anim Reprod Sci. 36: 211-225.

Epstein, H. and Herz, A. 1964. Fertility and birthweights of sheep in a subtropical environment. Zeitschrift f. Tier-züchtung u. Züchtungsbiologie. 80(1-4): 232-266.

Eric, W.S. and Herman, H.A. 1944. Seasonal variation in semen quality of some Missouri dairy bulls. J Dairy Sci. 916: 303-310.

Evans, G., Maxwell, W. and Salamon, S. 1987. Artificial insemination of sheep and goats. Sydney, Australia: Butterworths. 158-159.

Farshad, A., Amidi, F., Koohi Khor, A. and Rashidi, A. 2011. Effect of cholesterol-loaded-cyclodextrin in presence and absence of egg yolk during freezing step on quality of Markhoz buck's spermatozoa. Asian-Aust J Anim Sci. 24: 181-189.

Fahy, G.M. 2010. Cryoprotectant toxicity neutralization. Cryobiology 60: S45–S53.

Fitzgerald, J. 1997. Applied reproductive physiology of the ram. In: Current therapy in large animal theriogenology. R.S. Youngquist (ed.). Philadelphia: WB Saunders. 129-137.

Fukushima, F.B., Malm, C., Henry, M., Gheller, V.A., Serakides, R., Neves, M.M., Macedo, S.P., Figueiredo, M.S., Andrade, M.E.J., Chaves, M.S., Silva, M.X., Rezende, C.M.F. and Melo, E.G. 2010. Site of intrauterine artificial insemination in the bitch does not affect sperm distribution within the uterus. Reprod Dom Anim. 45(6): 1059-1064.

Gebre, M. Y. 2007. Reproductive traits in Ethiopian male goats, with special reference on breed and nutrition. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala. 72: 56-58.

Gherardi, P.B., Lindsay, D.R. and Oldham, C.M. 1980. Testicular size in rams and flock fertility. Proc Aust Sot Anim Prod. 13: 48–50.

Gil, J., Lundeheim, N., Söderquist, L. and Rodríguez Martínez, H. 2003. Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology 59: 1241-1255.

Gill, J., Rodriguez-Irazoqui, M., Lundeheim, N., Söderquist, L. and Rodríguez-Martínez, H. 2003. Fertility of ram semen frozen in Bioexcell® and used for cervical artificial insemination. Theriogenology 59: 1157-1170.

Goonewardene, L.A., Whitmore, W., Jaeger, S., Borchert, T., Okine, E., Ashmawy, O. and Emond, S. 1996. Effect of prebreeding maintenance diet on subsequent reproduction by artificial insemination in alpine and Saanen goats. Theriogenology 48(1): 151-59.

Gordon, I. 1997. Controlled Reproduction in Sheep and Goats. 1st ed. Cambridge Wallingford: University Press. 115pp.

Grayling, J.P.C. and Grobbelaar, J.A.N. 1983. Seasonal variation in semen quality of Boer and Angora goat rams using different collection techniques. S Afr J Anim Sci. 13: 250-252.

Gualtieri, R., Boni, R., Tosti, E., Zagami, M. and Talevi, R. 2005. Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium *in vitro*. Reproduction 129: 51–60.

Hawk, H.W. 1972. Progestagen induced sperm breakage in the sheep vagina. J Anim Sci. 34: 795-798.

Helal, A., Hashem, A.L.S., Abdel-Fattah, M.S. and El-Shaer, H.M. 2010. Effect of heat stress on coat characteristics and physiological responses of Balady and Damascus goats in Sinai, Egypt. American-Eurasian J Agric & Environ Sci. 7: 60-69.

Hibbert, L.M., Rodrigues, H.D., Noble, R.C., Vig, N.M. and Goyal, H.O. 1986. Effects of age and season on sperm abnormalities in Nubian goats. Anat Histol Embryol. 15: 173.

Hill, J. 1996. Maximizing the results of goat embryo transfer programs. Proceeding of the American Association of Small Ruminant Practitioners. Research Symposium on Health and Disease. Nashville, TN. 120.

Hiroe, K. and Tomizuka, T. 1966. Effect of high environmental temperature on the semen production in domestic animals. Jpn Bull Natl Inst Anim Ind. 9: 27–35.

Hoekstra, H.E. 2006. Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity 97: 222–234.

Holt, W.V. 2000[a]. Basic aspects of frozen storage of semen. Anim Reprod Sci. 62: 3-22.

Holt, W.V. 2000[b]. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. Theriogenology 53: 47-58.

Holtz, W., Sohnrey, B., Gerland, M., and Driancourt, M.A. 2008. Ovysych synchronization and fixed-time insemination in goats. Theriogenology 6: 785-792.

Hunter, R.H.F., Barwise, L. and King, R. 1982. Sperm transport, storage and release in the sheep oviduct in relation to the time of ovulation. Br Vet J. 138: 225-232.

Husain, S.S., Amin, M.R. and Islam, A.B.M.M. 1998. Goat production and its breeding strategy in Bangladesh. Proceedings of the 1st National Workshop on Animal Breeding, Nov. 26, BAU, Mymensingh, Bangladesh. 17-36.

Husain, S.S., Horst, P. and Islam, A.B.M.M. 1996. Study on the growth performance of Black Bengal goats in different periods. Small Rumin Res. 21: 165-171.

Iritani, A. and Nishikawa, Y. 1961. Studies on the egg-yolk coagulating factors in goat semen: II Properties of the coagulating factor and influential conditions for coagulation. In: Proc. Silver Jubilee Lab. Anim. Husbandry, Kyoto University. 97–104.

Iritani, A. and Nishikawa, Y. 1963. Studies on the egg-yolk coagulating factor in goat semen. V. Purification of the egg-yolk coagulating enzyme. Jpn J Anim Reprod. 8: 118–121.

Iritiani, A., Nishikawa, U. and Fukuhara, R. 1961. Studies on the egg-yolk coagulating factor in goat sperm. I. Localization of coagulating factors and decline of pH following coagulating. In: Proceedings of the Silver Jubilee Lab. Anim. Husbandry. 89–96.

Ishwa, A.K. and Memon, M.A. 1996. Embryo transfer in sheep and goats: a review. Small Rumin Res. 19: 35-43

Ismaya. 2003. Influence of the female reproductive tract on the motility and morphological characteristics of ram spermatozoa. PhD thesis, James Cook University. 93-115.

Jackson, I.J. 1994. Molecular and developmental genetics of mouse coat color. Annu Rev Genet. 28: 189-217.

Janett, F., Thun, R., Niederer, K., Burger, D. and Hässig, M. 2003. Seasonal changes in semen quality and freezability in the Warmblood stallion. Theriogenology 60: 453–461.

Jeulin, C., Lewin, L.M., Chevrier, C. and Schoevaert Brossault, D. 1996. Changes in flagellar movement of rat spermatozoa along the length of the epididymis: Manual and computer-aided image analysis. Cell Motil Cytoskeleton. 35(2): 147-161.

Jimenez, C. F. 1987. Effects of Equex STM and equilibration time on the pre-freeze and post thaw motility of equine epididymal spermatozoa. Theriogenology 28: 773–782.

Jones, R.C. and Martin, I.C.A. 1973. The effects of dilution, egg yolk and cooling to 5°C in the ultrastructure of ram spermatozoa. J Reprod Fertil. 35: 311–320.

Julian, S.M., Adolfo, T.D., Antonio, P.P., Jesus, D., Amelia, G.B. and Antonio, L.S. 2006. Effect of egg yolk concentration on cryopreserving Spanish ibex (*Copra pyrenica*) epididymal spermatozoa. Theriogenology 66: 1219-1226.

Karagiannidis, A., Varsakeli, S. and Karatzas, G. 2000. Characteristics and seasonal variations in the semen of Alpine, Saanen and Damascus goat bucks born and raised in Greece. Theriogenology 53: 1285-1293.

Karsch, F.J., Bittnian. E.X., Foster, D.L., Goodman, R.L., Legan, S.J. and Robinson, J.E. 1984. Neuro-endocrine basis of seasonal reproduction. Recent Prog Horm Res. 40: 185-232.

Khan, R.A. 1999. A quantitative study on semen characteristics of Black Bengal Buck. M.S. Thesis, Bangladesh Agricultural University, Mymensingh.

Killeen, I.D., Caffrey, G.J. and Holt, N.1982. Fertility of ewes following intra-uterine insemination with the aid of a laparoscope. Proc. Aust. Soc. Reprod. Biol. Annu. Conf., Sydney, N.S.W., Abstr. No. 104.

King, G.J. 1993. Reproduction in domestic animals. In: World Animal Science Series. B.V. London, New York: Elsevia Science Publisher.

King, L.M., Brillard, J.P., Garrett, W.M., Bakst, M.R. and Donoghue, A.M. 2002. Segregation of spermatozoa within sperm storage tubules of fowl and turkey hens. Reproduction 123: 79–86.

Klein, U., Gimpl, G. and Fahrenholz, F. 1995. Alteration of the myometrial plasma membrane cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin receptor. Biochemistry 34: 13784-13793.

Kozdrowski, R., Dubiel, A., Bielas, W. and Dzięcioł, M. 2007. Two protocols of cryopreservation of goat semen with the use of computer-assisted semen analysis system. Acta Vet Brno. 76: 601-604.

Kulaksiz, R. and Daşkin, A. 2012. Reproductive performance of primiparous and mutiparous Saanen goats after laparoscopic intrauterine insemination: a field study. Turk J Vet Anim Sci. 36: 201-204.

Kundu, C.N., Chakraborty, J., Dutta, P., Bhattacharyya, D., Ghosh, A. and Majumder, G.C. 2000. Development of a simple sperm cryopreservation model using a chemically defined medium and goat cauda epididymal spermatozoa. Cryobiology 40: 117-125.

Langendijk, P., Bouwman, E.G., Kidson, A., Kirkwood, R.N., Soede, N.M. and Kemp, B. 2002. Role of myometrial activity in sperm transport through the genital tract and in fertilization in sows. Reproduction 123: 683-690.

Latham, K.E. and Westhusin, M.E. 2000. Nuclear transplantation and cloning in mammals. Meth Mol Biol. 136: 405-425.

Laubser, P.P., van Niekerk, C.H., Botha, L.J.J., 1982. Seasonal changes in sexual activity and sperm quality in the Angora ram: 1. Libido and male hormone concentrations. S Afr J Anim Sci. 13(2): 131–133.

Leboeuf, B., Manfredi, E., Boue, P., Piacère, A., Brice, G., Baril, G., Broqua, C., Humblot, P. and Terqui, M. 1998. Artificial insemination of dairy goats in France. Livest Prod Sci. 55: 193–202.

Leboeuf, B., Restall, B. and Salamon, S. 2000. Production and storage of goat semen for artificial insemination. Anim Reprod Sci. 62: 113–141.

Lehloenya, K.C. and Greyling, J.P.C. 2010. The ovarian response and embryo recovery rate in Boer goat does following different superovulation protocols, during the breeding season. Small Rumin Res. 88: 38–43.

Lincoln, G.A. 1978. The temporal relationship between plasma levels of FSH and LH in the ram. J Reprod Fert. 53: 31-37.

Lincoln, G.A. and Kay, N.B. 1979. Effects of season on the secretion of LH and testosterone in intact and castrated red deer stags (*Cervus elaphus*). J Reprod Fert. 55: 75-80.

Loskutoff, N.M., Sirnmons, H.A., Goulding, M., Thompson, G., De Jongh, T. and Simmons, L.G. 1996. Species and individual variations in cryoprotectant toxicities and freezing resistances of epididymal sperm from African antelope. Anim Repro Sci. 42: 527-535.

Maina, V.A., Chaudhari, S.U.R. and Mshelia, G.D. 2006. Spermiogram of white ecotype of sahel bucks in Maiduguri Metropolis. Pak J Biol Sci. 9: 307-309.

Martin, J.C., Klug. E. and Gunzel, A.R. 1979. Centrifugation of stallion semen and its storage in large volume straws. J Reprod Fert. Suppl. 27: 47–51.

Martin, G.B. and Walkden-Brown, S.W. 1995. Nutritional influences on reproduction in mature male sheep and goats. Reprod Fertil Supple. 49: 437–449.

Martínez-Rojero, R.D., Hernández, J., Reyna, L. and Santos, R. 2007. Intrauterine insemination with frozen-thawed semen in Creole goats synchronized in estrous during the non breeding season. Res J Anim Sci. 1(3): 76-80.

Maxwell, W.M.C., Butler, L.G. and Wilson, H.R. 1984. Intra-uterine insemination of ewes with frozen semen. J Agric Sci. 102: 233-235.

Maxwell, W.M.C., 1986. Artificial insemination of ewes with frozen-thawed semen at a synchronized oestrus. 2. Effect of dose of spermatozoa and site of intrauterine insemination on fertility. Anim Reprod Sci. 10: 309-316.

Mazur, P. 1984. Freezing of living cells: mechanisms and implications. Am J Physiol cell Physiol. 247: C125-C142.

Mazur, P. and Kleinhans, F.W. 2008. Relationship between intracellular ice formation in oocytes of the mouse and Xenopus and the physical state of the external medium-a revisit. Cryobiology 56: 22–27.

McKelvey, W.A.C., Robinson J.J. and Aitken, R.P. 1985. A simplified technique for the transfer of ovine embryo by laparoscopy. Vet Record. 117: 492–494.

Miller, D.J., Demers, J.M., Braundmeier, A.G. and Behrens, M.L. 1998. The use of two fluorescent dyes to identify sperm in a competitive binding assay to oocytes. J Androl. 19(6): 650-656.

Mirarchi, R.E., Howland, B.E., Scanion, P.F., Kirkpatrick, RX. and Sanford, L.M. 1978. Seasonal variation in plasma LH, FSH, prolactin and testosterone concentrations in adult male white-tailed deer. Can J Zoo. 56: 121-127.

Mobini, S., Heath, A.M. and Pugh, D.G. 2002. Theriogenology of Sheep and Goats. In: Sheep and Goat Medicine. 1st ed. D.G. Pugh (ed.). USA: W.B. Saunders company. 129-186.

Mohri, H., Hasegawa, S. and Masaki, J. 1970. Seasonal change in glycerol kinase activity of goat spermatozoa. Biol Reprod. 2: 352-355.

Mortimer, S.T. 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. Hum Reprod Update 3: 403-439.

Muhuyi, E.Z., Drobnis, E.Z., Nelxon, E.A. and Lin, T.Y. 1992. Season, breed and age influence on production and freezability of dairy goat semen (Abstract). In: Proc. 3rd Int. conf. Goat Prod. Disease, Tucson, Arizona, USA. 283

Mukherjee, D.P., Roy, A. and Bhattacharya, P. 1953. The effect of feeding thyroprotein on semen quality and on some physiological conditions of goats: I. Effects on reaction time and semen characteristics. Indian J Vet Sci. 23: 1–24.

Murase, T., Imaeda, N., Yamada, H. and Miyazawa, K. 2007. Seasonal changes in semen characteristics, composition of seminal plasma and frequency of acrosome reaction induced by calcium and calcium ionophore A23187 in Large White boars. J Reprod Dev. 53: 853-864.

Niu, Y., Greube, A., Ji, W. and Jewgenowa, K. 2006. The application of in vitro sperm competition test to evaluate the impact of ZP-derived peptides on fertilization capacity of cat sperm. Theriogenology 66: 989–995.

Nunes, J.F., Corteel, J.M., Combarnous, Y. and Baril, G. 1982. Role of seminal plasma in the in vitro survival of goat sperm. Reprod Nutr Dev. 22: 611–620.

Nur, Z., Dogan, I., Gunay, U. and Soylu, M.K. 2005. Relationships between sperm membrane integrity and other semen quality characteristics of the semen of Saanen goat bucks. Bull Vet Inst Pulawy. 49: 183-187.

Ogebe, P.O., Ogunmodede, B.K. and McDowell, L.R. 1996. Behavioral and physiological responses of Nigerian dwarf goats to seasonal changes of the humid tropics. Small Rumin Res. 22: 213-217.

Ortavant, R., 1977. Photoperiodic regulation of reproduction in the sheep. In: Management of Reproduction in Sheep and Goats Symposium, Madison. 58–71.

Ortman, K. and Rodriguez-Martin, H. 1994. Membrane damage during dilution, cooling and freezing-thawing of boar spermatozoa packaged in plastic bags. Vet Med A-Zbl Vet A–Physiol. 41: 37–47.

Parrish, J.J. and Foote, R.H. 1985. Fertility differences among male rabbits determined by heterospermic insemination of fluorochrome-labeled spermatozoa. Biol Reprod. 33: 940-949.

Parrish, J.J. and Foote, R.H. 1986. Fertility of cooled and frozen rabbit sperm measured by competitive fertilization. Biol Reprod. 35: 253-257.

Paul, S., Khandoker, M.A.M.Y., Moinuddin, M.A. and Paul, R.C. 2011. Characterization of Black Bengal goat. J Bangladesh Agril Univ. 9: 61–66.

Peña, A. and Linde-Forsberg, C. 2000. Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 54: 859–875.

Peña, A., Lugilde, L.L., Barrio, M., Herradón, P.G. and Quintela, L.A. 2003. Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca^{2+} concentration of dog spermatozoa. Theriogenology 59: 1725-1739.

Perkins, N.R., Hill, J.R. and Pedrana, R.G. 1996. Laparoscopic insemination of frozen-thawed semen into one or both uterine horns without regard to ovulation site in synchronized merino ewes. Theriogenology 46(3): 541-545.

Pellicer-Rubio, M.T., Magallon, T. and Combarnous, Y. 1997. Deterioration of goat sperm viability in milk extenders is due to a bulbourethral 60 kDa glycoprotein with triglyceride lipase activity. Biol Reprod. 57: 1023–1031.

Peskovatskov, A.P., Vasitjev, G.I. and Dzumagahiev, M. 1974. First results of artificial insemination with frozen semen. Zhivotnovodstvo 8: 71–72.

Peterson, K., Kappen, M.A.P.M., Ursem, P.J.F., Nöthling, J.O., Colenbrander, B. and Gadella, B.M. 2007. Microscopic and flow cytometric semen assessment of Dutch AI-bucks: Effect of semen processing procedures and their correlation to fertility. Theriogenology 67: 863–871.

Preston, B.T., Stevenson, I.R., Pemberton, J.M. and Wilson, K. 2001. Dominant rams lose out by sperm depletion. Nature 409: 681–682.

Puglisi, R., Krvavac, L., Bonacina, C. and Galli, A. 2010. *In vitro* competitive binding index using fluorochrome-labelled spermatozoa for predicting bull fertility. Zygote 18: 281–291.

Purdy, P.H. 2006. A review on goat sperm cryopreservation. Small Rumin Res. 63: 215–225.

Pursel, V.G., Schulman, L.L. and Johnson, L.A. 1978. Effect of Orvus ES Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. J Anim Sci. 47: 198–202.

Quinlivan, T.D. and Robinson, T.J. 1969. Numbers of spermatpzoa in the genital tract after artificial insemination of progestagen-treated ewes. J Reprod Fertil. 19: 73-86.

Ramon-Ugalde, J.P., Folch, J., Cocero, M.J., Piña-Aguilar, R.E. and Alabart, J.L. 2008. Embryo recovery from the oviduct in superovulated ewes: a method to improve MOET systems. Czech J Anim Sci. 53: 145–151.

Remy, B., Baril, G., Vallet, J. C., Dufour, R., Chouvet, C., Saumande, J., Chupin, D. and Beckers, J. F. 1991. Are antibodies responsible for a decreased superovulatory response in goats which have been treated repeatedly with porcine follicle-stimulating hormone? Theriogenology 36: 389-399.

Restall, B.J. 1991. Goat production in the Asian humid tropics. In: Goat Production in Asian Humid Tropics. B.W. Norton and D. Saithanoo (eds.). Proc. Int. Conf., Hat Yai, Thailand. 74–83.

Revell, S.G. and Mrode, R.A. 1994. An osmotic resistance test for bovine semen. Anim Reprod Sci. 36: 77-86.

Ritar, A.J., Ball, P.D., Black, T., Jackson, R.B., O'May, P., Hazlewood, F. and Graham, G. 1987. AI of Cashmere goats: effect of CIDR or sponge, dose of frozen-thawed semen and time of cervical or laparoscopic insemination. Aus Soc Reprod Biol. 28.

Ritar, A.J., Ball, P.D. and O'May, P.J. 1990[a]. Artificial insemination of Cashmere goats: effects on fertility and fecundity of intravaginal treatment, method and time of insemination, semen freezing process, number of motile spermatozoa and age of females. Reprod Fertil Dev. 2: 377–384.

Ritar, A.J., Ball, P.D. and O'May, P.J. 1990[b]. Examination of methods for the deep freezing of goat semen. Reprod Fertil Dev. 2: 27–34.

Ritar, A.J., Maxwell, W.M.C., and Salamon, S. 1984. Ovulation and LH secretion in the goat after intravaginal progestagen sponge-PMSG treatment. J Reprod Fertil. 72: 559-563.

Ritar, A.J. and Salamon, S. 1982. Effects of seminal plasma and of its removal and egg yolk in the diluent on the survival of fresh and frozen-thawed spermatozoa of Angora goat. Aust J Biol Sci. 35: 305–312.

Ritar, A.J., Salamon, S., Ball, P.D. and O'May, P.J. 1989. Ovulation and fertility in goats after intravaginal device-PMSG treatment. Small Rumin Res. 2: 323-331.

Rodriquez, F., Baldassarre, H., Simonetti, J., Aste, F. and Ruttle, J.L. 1988. Cervical versus intrauterine insemination of ewes using fresh or frozen semen diluted with aloe vera gel. Theriogenology 30(5): 843-854.

Rota, A., Ström, B., Linde-Forsberg, C., and Rodriguez-Martinez, H., 1997. Effects of STM paste on viability of frozen thawed dog spermatozoa during in vitro incubation at 38°C. Theriogenology 47: 1093–1101.

Roy, A. 1957. Egg yolk coagulating enzyme in the semen and Cowper's gland of the goat. Nature 179: 318–319.

Rudenko, S.V., Gapochenko, S.D. and Bondarenko, V.A. 1984. Effect of glycerol on the capacitance and conductivity of bilayer lipid membranes. Biophysics 29: 245-249.

Saacke, R.G. and White, J.M. 1972. Semen quality tests and their relationship to fertility. In: Proceedings of the 4th Technical Conference on Artificial Insemination and Reproduction, National Association of Animal Breeders. February 17-19, 1972, Columbia. MO. USA. 22–27.

Salamon, S. and Ritar, A.J. 1982. Deep freezing of Angora goat semen: effects of diluent composition and method and rate of dilution on survival of spermatozoa. Aust J Biol Sci 35: 295–303.

Salvador, I., Yániz, J., Viudes-de-Castro, M.P., Gómez, E.A. and Silvestre, M.A. Effect of solid storage on caprine semen conservation at 5°C. 2006. Theriogenology 66: 974–981.

Scenna, F.N., Hockett, M.E., Towns, T.M., Saxton, A.M., Rohrbach, N.R., Wehrman, M.E. and Schrick, F.N. 2005. Influence of a prostaglandin synthesis inhibitor administered at embryo transfer on pregnancy rates of recipient cows. Prostaglandins Other Lipid Mediat. 78(1-4): 38-45.

Scott, M.A. 2000. A glimpse at sperm function *in vivo*: sperm transport and epithelial interaction in the female reproductive tract. Anim Reprod Sci. 60: 337-348.

Shin, S.T., Jang, S.K., Yang, H.S., Lee, O.K., Shim, Y.H., Choi, W.I., Lee, D.S., Lee, G.S., Cho, J.K. and Lee, Y.W. 2008. Laparoscopy vs. laparotomy for embryo transfer to produce transgenic goats (*Capra hircus*). J Vet Sci. 9(1): 103–107.

SimplÍcio, A.A., Riera, G.S., Nelson, E.A. and Pant, K.P. 1982. Seasonal variation in seminal and testicular characteristics of Brazilian Somali rams in the hot semi-arid climate of tropical northeast. Brazil J Reprod Fert. 66: 735-738.

Singh, M.P., Sinha, A.K. and Singh, B.K. 1995. Effect of cryoprotectants on certain seminal attributes and on the fertility of buck spermatozoa. Theriogenology 43: 1047-1053.

Sohnrey, B. and Holtz, W. 2005. Technical Note: Transcervical deep corneal insemination of goats. J Anim Sci. 83: 1543-1548.

Sönmez, M. and Demirci, E. 2004. The effect of ascorbic acid on the freezability of ram semen diluted with extenders containing different proportions of glycerol. Turk J Vet Anim Sci. 28: 893-899.

Sukcharoen, N., Keith, J., Irvine, D.S., and Aitken, R.J. 1996. Prediction of the in vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. Hum Rep. 11: 1030–1034.

Summermatter, P. and Flukiger, A. 1982. Buck semen processing during off breeding season (Abstract). In: Proc. 3rd Int. Conf. Goat Prod. Disease, Tucson, AZ, USA. 284.

Sundararaman, M.N. and Edwin, M.J. 2008. Changes in motility characteristics of goat spermatozoa during glycerol-equilibration and relevance to cryopreservation. Asian J Cell Biol. 3: 22-33.

Suriyasomboon, A., Lundeheim, N., Kunavongkrit, A., and Einarsson, S. 2004. Effect of temperature and humidity on sperm production in Duroc boars under different housing systems in Thailand. Livest Prod Sci. 89: 19–31.

Suyadi, Sohnrey, B. and Holtz, W. 2000. Transcervical embryo collection in Boer goats. Small Rumin Res. 36: 195-200.

Taggart, D.A., Leigh, C.M., Steele, V.R., Breed, W.G., Temple-Smith, P.D. and Phelan, J. 1996. Effect of cooling and cryopreservation on sperm motility and morphology of several species of marsupial. Reprod Fertil Dev. 8: 673-679.

Tejada, R.I., Mitchell, J.C., Norman, A., Marik, J.J. and Friedman, S. 1984. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril. 42: 87-91.

Thom, E.C. 1959. The discomfort index. Weatherwise 12:57–59.

Tretipskul, C., Buranaamnuay, K., Koonjaenak, S., Tummaruk, P. and Techakumphu, M. 2010. The use of computer-assisted sperm analysis (CASA) for discriminating a series of motility pattern of frozen-thawed boar semen. Thai J Vet Med. 40(1): 25-30.

Trounson, A.O. and Moore, N.W. 1974. Fertilization in the ewe following multiple ovulation and uterine insemination. Aust J Biol Sci. 27: 301-04.

Tsutsui, T., Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Murai, M., Anzai, M. and Hori, T. 2000. Unilateral intrauterine horn insemination of fresh semen in cats. J Vet Med Sci. 62(12): 1241-1245.

Tuli, R.K. and Holtz, W. 1992. The effect of season on seminal characters in Boer goat bucks in the Northern temperate zone (Abstract). In: Recent Advances in Goat Production. Proc. papers presented at 5th Int. Conf. on Goats. New Delhi. 1195.

Tuli, R.K., Holtz, W., 1994. Effect of glycerolization procedure and removal of seminal plasma on post-thaw survival and GOT-release from Boer goat spermatozoa. Theriogenology 42: 547–555.

Tummaruk, P., Sumransap, P., Techakumphu, M. and Kunavongkrit, A. 2007. Distribution of spermatozoa and embryos in the female reproductive tract after unilateral deep intra uterine insemination in the pig. Reprod Domest Anim. 42: 603-609.

Tummaruk, P. and Tienthai, P. 2010. Number of spermatozoa in the crypts of the sperm reservoir at about 24 h after a low-dose intrauterine and deep intrauterine insemination in sows. Reprod Dom Anim. 45: 208–213.

Verstegen, J., Iguer-Ouada, M. and Onclin, K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57: 149-179.

Waide, Y., Niwa, T. *and* Asanuma, R. 1977. Studies on preservation of liquid and frozen semen of domestic animals. III. Viability and fertility of frozen goat spermatozoa. Jpn J Anim Reprod. 23: 129-137.

Warwick, B.L., Barry, R.O. and Horlacher, W.R. 1934. Results of mating rams to Angora female goats. In: Proceeding of the. 27th Annu. Meet. Am. Sot. Anim. Prod. 225-227.

Waterhouse, K.E., Hofmo, P.O., Tverdal, A. and Miller, R.R.Jr. 2006. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction 131: 887-894.

Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev. 7: 871–891.

Williams, W.W. 1920. Technique of collecting semen for laboratory examination with a review of several diseased bulls. Cornell Vet. 10: 87–94.

Yamashiro, H., Han, Y.J., Sugawara, A., Tomioka, I., Hoshino, Y. and Sato, E. 2007. Freezability of rat epididymal sperm induced by raffinose in modified Krebs–Ringer bicarbonate (mKRB) based extender solution. Cryobiology 55: 285–294.

Yokoki, Y. and Ogasa, A. 1977. Effects of hypothermia on semen production in goats. Jpn J Anim Reprod. 23(3): 93–98.

Zahn, F.S., Papa, F.O. and Dell'Aqua Jr., J.A. 2002. Cholesterol incorporation on equine sperm membrane: effects on post-thaw sperm parameters and fertility. Theriogenology 58: 237-240.

Zamiri, M.J. and Heidari, A.H. 2006. Reproductive characteristics of Rayini male goats of Kerman province in Iran. Ani Reprod Sci. 96: 176-185.

Zarazaga, L.A., Guzmán, J.L., Domínguez, C., Pérez, M.C. and Prieto, R. 2009. Effects of season and feeding level on reproductive activity and semen quality in Payoya buck goats. Theriogenology 71: 1316–1325.

APPENDIX

List of proceedings

1. **Anakkul, N.**, Suwimonteerabutr, J., Tharasanit, T., Khunmanee, S., Diloksumpan, P., Thanomsuksinchai, N., Panyaboriban, S., Phutikanit, N. and Techakumphu, M. 2013. Artificial insemination and embryo transfer can be used for genetic improvement and disease prevention in goat industry. RGJ-Ph.D. Congress XIV, 5-7 April 2013, Jomtien Palm Beach Resort, Pattaya, Chonburi.
2. **Anakkul, N.**, Suwimonteerabutr, J., Khunmanee, S., Thanomsuksinchai, N., Panyaboriban, S., Tharasanit, T. and Techakumphu, M. 2012. Laparoscopic artificial insemination with frozen semen and embryo transfer with early embryonic stage in goat. RGJ Seminar Series LXXXVII: "From Life Science to One Health". 17 May 2012, Faculty of Vet. Science, Chulalongkorn University, p.21 **(Best oral presentation)**
3. **Anakkul, N.**, Suwimonteerabutr, J., Panyaboriban, S., Khunmanee, S., Thanomsuksinchai, N., Tharasanit, T. and Techakumphu, M. 2012. Production of Black Skin Goat Using Embryo Transfer Technique. Proceeding of the 1st Asia Dairy Goat Conference, 9–12 April 2012, University Putra Malaysia, Kuala Lumpur, Malaysia, p.228-229
4. **Anakkul, N.**, Suwimonteerabutr, J., Khunmanee, S., Diloksumpan, P., Tharasanit, T. and Techakumphu, M. 2011. Unilateral and bilateral laparoscopic insemination of frozen goat semen. Proceeding of the 10th Chulalongkorn University Veterinary Annual Conference, 21-22 April 2011, Faculty of Vet. Science, Chulalongkorn University, p.S39
5. **Anakkul, N.**, Suwimonteerabutr, J., Khunmanee, S., Diloksumpan, Virakul, P., Tharasanit, T. and Techakumphu, M. 2011. Artificial insemination in goat in Thailand from bench to industry. RGJ Seminar Series LXXX: "Innovations for Animal Health and Production II", 20 April 2011, Faculty of Vet. Science, Chulalongkorn University, p.16

6. **Anakkul, N.**, Suwimonteerabutr, J., Khunmanee, S., Diloksumpan, P., Virakul, P., Tharasanit, T. and Techakumphu, M. 2011. Artificial insemination in goat in Thailand from bench to industry. RGJ-Ph.D. Congress XII, 1-3 April 2011, Jomtien Palm Beach Resort, Pattaya, Chonburi, p.333 **(Best poster presentation)**
7. **Anakkul, N.**, Suwimonteerabutr, J., Khunmanee, S., Diloksumpan, P., Promthep, K., and Techakumphu, M. 2010. Semen quality from consecutive collection of goat in Thailand. The 13th Association of Institutions for Tropical Veterinary Medicine (AITVM) Conference, 23-25 August, 2010, Bangkok, Thailand, p.207
8. **Anakkul, N.**, Suwimonteerabutr, J., Phutikanit, N. and Techakumphu, M. 2010. Cryopreservation of Goat Semen in Extenders Supplemented with Different Glycerol Concentrations RGJ Seminar Series LXXI "Perspectives and Innovation in Veterinary Biosciences", 24th February 2010, Faculty of Vet. Science, Chulalongkorn University, p.30
9. **Anakkul, N.**, Khunmanee, S., Diloksumpan, P., Promthep, K., Suwimonteerabutr, J. and Techakumphu, M. 2010. Cryopreservation of goat semen in extenders supplemented with different concentrations of Equex STM Paste. Proceeding of the 9th Chulalongkorn University Veterinary Annual Conference, 1st April 2010, Faculty of Vet. Science, Chulalongkorn University, p.135
10. **Anakkul, N.**, Suwimonteerabutr J., Phutikanit N. and Techakumphu M. 2009. Assessment of semen quality in goat using CASA techniques. The 6th annual meeting of the Asian Reproductive Biotechnology Society, 16-20 November, 2009, Seam Reap City, Cambodia, p.137
11. **Anakkul, N.**, Suwimonteerabutr J., Singlor, J., Phutikanit N. and Techakumphu, M. 2009. Effect of Equex STM Paste on goat semem cryopreservation. Proceeding of the 8th Chulalongkorn University Veterinary Annual Conference, 3rd April 2009, Faculty of Vet. Science, Chulalongkorn University, p.81

BIOGRAPHY

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Publications

1. **Anakkul, N.**, Suwimonteerabutr, J., Singlor, J., Phutikanit N., Tharasanit, T. and Techakumphu, M. 2011. Effect of Equex STM Paste on the quality and motility characteristics of post thawed cryopreserved goat semen. Thai J of Vet Med. 41(3). 345-351.
2. **Anakkul, N.**, Suwimonteerabutr, Tharasanit, T., Phutikanit N., Singlor, J. and Techakumphu, M. 2013. Glycerol concentration affects on quality and longevity of post-thaw goat semen. Thai J of Vet Med.