การแช่แข็งน้ำเชื้อ การกระตุ้นเพิ่มการตกไข่ด้วย เอฟ เอส เอช ระบบปล่อยช้า
และการย้ายฝากตัวอ่อนในแกะในประเทศไทย

นายสฤษฏ์วิชญ์ ปัญญาบริบาลบ์





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# SEMEN CRYOPRESERVATION, SUPEROVULATION USING A SLOW FSH RELEASING SYSTEM AND EMBRYO TRANSFER IN SHEEP IN THAILAND

Mr. Saritvich Panyaboriban





A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
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สฤษฏ์วิชญ์ ปัญญาบริบาลบ์ : การแช่แข็งน้ำเชื้อ การกระตุ้นเพิ่มการตกไข่ด้วย เอฟ เอส เอช ระบบปล่อยช้าและการย้ายฝากตัวอ่อนในแกะในประเทศไทย (SEMEN CRYOPRESERVATION, SUPEROVULATION USING A SLOW FSH RELEASING SYSTEM AND EMBRYO TRANSFER IN SHEEP IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.สพ. ดร. มงคล เตชะกำพุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. น.สพ. ดร. ธีรวัฒน์ ธาราศานิต, อ. น.สพ. ดร. ธีรวัฒน์ สว่างจันทร์อุทัย, 90 หน้า.

วิทยานิพนธ์ฉบับนี้มีวัตถุประสงค์เพื่อศึกษาผลการประยุกต์ใช้เทคโนโลยีชีวภาพทางระบบสืบพันธุ์โดยเทคนิคการผสมเทียมและการย้ายฝากตัวอ่อนเพื่อการเพิ่มประสิทธิภาพทางพันธุกรรมและการสืบพันธุ์ของแกะ รวมถึงการนำไปใช้จริงในระดับฟาร์มในประเทศไทย ฤดูกาลและสิ่งแวดล้อมอาจส่งผลกระทบต่อคุณภาพและคุณลักษณะของน้ำเชื้อจากพ่อพันธุ์แกะนำเข้าจากต่างประเทศ จากกรณีศึกษาพบว่าคุณภาพน้ำเชื้อและขนาดวงรอบของถุงหุ้มอัณฑะมีความผันแปรตามฤดูกาล มีความสัมพันธ์กับอุณหภูมิ ความชื้น และปริมาณแสง พ่อพันธุ์แกะนำเข้ามีประสิทธิภาพทางการสืบพันธุ์สูงสุดอยู่ในระหว่างเดือนธันวาคมถึงมีนาคมของปี ในการศึกษาประสิทธิภาพของน้ำตาลหลายชนิดและ/หรือการใช้น้ำตาลร่วมกันสองชนิดต่อคุณภาพของน้ำเชื้อแช่แข็งในแกะ และทดสอบประสิทธิภาพการปฏิสนธิของน้ำเชื้อแช่แข็งในแกะที่ถูกดำเนินการแช่แข็ง พบว่าสูตรน้ำยาแช่แข็งน้ำเชื้อที่ประกอบด้วยน้ำตาลซูโครสร่วมกับทรีฮาโลสสามารถเพิ่มคุณภาพของน้ำเชื้อแช่แข็งหลังการละลายได้อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) เมื่อเปรียบเทียบกับการใช้น้ำตาลชนิดอื่นๆ หลังจากผสมเทียมในแกะด้วยน้ำเชื้อในสูตรที่ดีที่สุด พบว่าอัตราการตั้งท้องในแม่แกะภายหลังการผสมเทียมแบบส่องกล้องลาพาโลสโคปผ่านช่องท้อง ในแกะให้ประสิทธิภาพที่ดีเทียบเท่ากับการผสมเทียมด้วยน้ำเชื้อสด (ร้อยละ 82 และ 84 ตามลำดับ) ในการศึกษาเปรียบเทียบอัตราการตกไข่ (โดยดูจากจำนวนคอร์ปัสลูเทีย) ไข่อ่อน ตัวอ่อน และอัตราการเก็บไข่ได้ระหว่างการกระตุ้นการตกไข่หลายใบชนิดฉีดเอฟเอสเอชน้อยครั้งที่ผสมด้วยไฮยาลูโรแนน (กลุ่ม S) กับโปรแกรมมาตรฐานที่ฉีดด้วยเอฟเอสเอชหลายครั้ง (กลุ่ม M) พบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญในการตอบสนองบนรังไข่ระหว่างกลุ่ม อย่างไรก็ตามพบว่าปริมาณตัวอ่อนคุณภาพดีโดยเฉลี่ยต่อตัวที่ผลิตได้จากการใช้โปรแกรมการกระตุ้นการตกไข่ซ้ำหลายใบในกลุ่ม S มีแนวโน้มที่สูงกว่ากลุ่ม M ภายหลังการย้ายฝากตัวอ่อนที่ผลิตได้จากกลุ่ม S ให้กับแกะตัวรับ มีอัตราการตั้งท้องที่สูงกว่ากลุ่ม M อย่างมีนัยสำคัญทางสถิติ (ร้อยละ 90.5 และ 78.9 ตามลำดับ) ท้ายที่สุดได้มีการนำเทคโนโลยีชีวภาพทางระบบสืบพันธุ์ชั้นสูงทั้งการผสมเทียมและการย้ายฝากตัวอ่อนที่ได้รับการศึกษาแล้วมาประยุกต์และนำไปใช้จริงในระดับฟาร์มแกะพันธุ์ดี พบว่าการใช้เทคโนโลยีเหล่านี้สามารถใช้ได้จริงอย่างมีประสิทธิภาพ

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| ภาควิชา | สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ | ลายมือชื่อนิสิต ..... |
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KEYWORDS: EMBRYO TRANSFER / LAPAROSCOPIC ARTIFICIAL INSEMINATION / SEMEN CRYOPRESERVATION / SHEEP / SPLIT-SINGLE FSH INJECTION / SUPEROVULATION

SARITVICH PANYABORIBAN: SEMEN CRYOPRESERVATION, SUPEROVULATION USING A SLOW FSH RELEASING SYSTEM AND EMBRYO TRANSFER IN SHEEP IN THAILAND. ADVISOR: PROF. DR. MONGKOL TECHAKUMPHU, D.V.M., D.E.A., Doctorat de 3e cycle, CO-ADVISOR: ASST. PROF. DR. THEERAWAT THARASANIT, D.V.M., Ph.D., DR. THEERAWAT SWANGCHAN-UTHAI, D.V.M., M.Sc., Ph.D., 90 pp.

The present thesis aims to utilize the assisted reproductive technology (ARTs) as Laparoscopic Insemination and Embryo Transfer (ET) for genetic and reproduction improvement in sheep and also to apply to commercial farm in Thailand. The seasonality and environmental may affect the semen traits in imported ram under tropical conditions. Semen qualities and scrotal circumference of an imported ram varied monthly related to season and environment especially during summer. Indeed, the ram's reproductive performances correlated with ambient temperature, humidity and day length. These results showed that the peak reproductive performances of imported ram were from December to March. Next, the effect of various and combination of sugars in semen extender for increasing frozen-thawed sperm quality have been investigated. Our findings showed that the combined sucrose and trehalose (ST) supplementation in semen freezing extender significantly improved frozen-thawed sperm qualities (motility, viability, longevity and acrosome integrity) when compared with other sugars ($P < 0.05$). The fertility rate after laparoscopic artificial insemination (LAI) with frozen-thawed ram semen with the best semen extender revealed the similar pregnancy rates compared with fresh semen (82% vs. 84%, respectively). Regarding the development of simplify superovulation technique in ewes, *in vivo* experiment was performed to compare ovarian responses and embryo yields by superovulatory stimulation with split-single FSH administration dissolved with hyaluronan (treated group; S) compared to a conventional method (multiple injections of FSH programs; control; M). The results showed no difference of the ovarian responses and embryo yields between the two groups. However, grade 1 and 2 recovered embryos in treated group tended to be higher than in the control group. Following the embryo transfer (ET), the pregnancy rates in S group (90.5%) were significantly higher ($P < 0.05$) than in M group (78.9%). Finally, the overall results obtained in this thesis were carried out to verify the possibility of application of both AI and ET techniques to commercial scale in sheep farm. It is found that the techniques proposed on this context can successfully translate from laboratory scale to commercial scale.

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| Department: | Obstetrics Gynaecology and Reproduction | Student's Signature | ..... |
| | | Advisor's Signature | ..... |
| Field of Study: | Theriogenology | Co-Advisor's Signature | ..... |
| Academic Year: | 2015 | Co-Advisor's Signature | ..... |

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| | |
|---|---|
| °C | degree celcius |
| $\chi^2$ | chi-square test |
| Acro | acrosome integrity |
| AI | artificial insemination |
| ARTs /ART | assisted reproductive technologies |
| ATP | adenosine triphosphate |
| AV | artificial vagina |
| BCS | body condition score |
| CASA | computer-assisted sperm analyzer |
| CAT | catalase |
| CATMOD | categorical model |
| CBC | complete blood count |
| CIDR | controlled internal drug-releasing device |
| CIDR-G | controlled internal drug-releasing device for goat |
| CL | corpus luteum |
| cm | centimeter |
| CPA | cryoprotective agent |
| CV | coefficients of variation |
| d | day |
| DLD | department of livestock development |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DPBS | Dubecco' s phosphate-buffered saline |
| eCG | equine chorionic gonadotropin |
| EE | electro-ejaculation |
| ET | embryo transfer |
| EY | egg yolk |
| FAO | Food and agriculture organization |

| | |
|---|---|
| FCS | fetal calf serum |
| FGA | fluorogestone acetate |
| FITC-PNA | fluorescein isothiocyanated peanut agglutinin |
| Fru | fructose |
| FSH | follicular stimulating hormone |
| FS | fructose and sucrose |
| FT | fructose and trehalose |
| Glu | glucose |
| GSH-Px | glutathione peroxidase |
| h | hour |
| hCG | human chorionic gonadotropin |
| HCl | hydrochloride salt |
| HA | hyaluronan/ hyaluronic acid |
| HA-FSH | FSH dissolved in HA |
| IACUC | Institutional animal care and use committee |
| IETS | International embryo transfer society |
| IM | intramuscular |
| IU | international unit |
| IVP | *in vitro* embryo production |
| kDa | kilodalton |
| Kg | kilogram |
| LAI | laparoscopic artificial insemination |
| LH | luteinizing hormone |
| LIN | linearity |
| m | month |
| M group | multiple FSH injection group |
| MAP | methyl-acetoxy progesterone |
| mOsm | milliosmole |
| mg | milligram |
| min | minute |
| mL | milliliter |

| | |
|---|---|
| mM | millimolar |
| mm | millimeter |
| Mo | motility |
| ng | nanogram |
| OPU | ovum pick-up |
| PBS | phosphate-buffered saline solution |
| pg | picogram |
| PG | propylene glycol |
| $PGF_{2\alpha}$ | prostaglandin $F_{2\alpha}$ |
| PMSG | pregnant mare serum gonadotropin |
| PVP | polyvinylpyrrolidone |
| ROS | reactive oxygen species |
| S group | split-single FSH injection group |
| s/sec | second |
| SAS | statistical analysis system |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| SEM | standard error |
| ST | sucrose and trehalose |
| STR | straightness |
| Su | sucrose |
| TC | tris-citric acid |
| THI | temperature-humidity index |
| Tre | trehalose |
| Tris | tris-(hydroxymethyl)-aminomethane |
| UTJ | uterotubal junction |
| v | versus |
| v/v | volume/ volume |
| VAP | average path velocity |
| VCL | curvilinear velocity |
| Via | viability |

| | |
|---|---|
| VSL | straight-line velocity |
| w/v | weight/volume |
| w | weight |

# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Importance and rationale

Small ruminants play an important role in agriculture and animal production in Thailand. There are many advantages to domesticate small ruminants in comparison with other farm animals such as bovine or swine, in terms of animal management, using small raising area, good environmental adaptation, short production cycle and competence production for various products, such as wool for clothing, medical suture, meat, and milk (Owen et al., 1978; Dubeuf et al., 2004; Devendra, 2013). The Food and Agriculture Organization (FAO) reported that the needs of consumable goat and sheep products have continually been increasing. In Asia, there are total of 577 million sheep and goats which are calculated to be approximately 59.7% of total head all over the world. Indeed, seven of the top ten countries of highly raising small ruminants are China, Pakistan, Turkey, Bangladesh, Iran, Mongolia and Indonesia (FAO, 2008). Nowadays, small ruminant agriculture in Thailand is very attractive due to their high profitability with low cost of production and the population is increasing meaning a high consumption as well. In Thailand, the small ruminant production is mainly located in the South (Livestock area 9) as seen in figure 1A & 1B. Therefore, the need of breeding of suitably genetic-adapted sheep and goats is necessary, principally via breeding selection combined with assisted reproductive technology (ART).

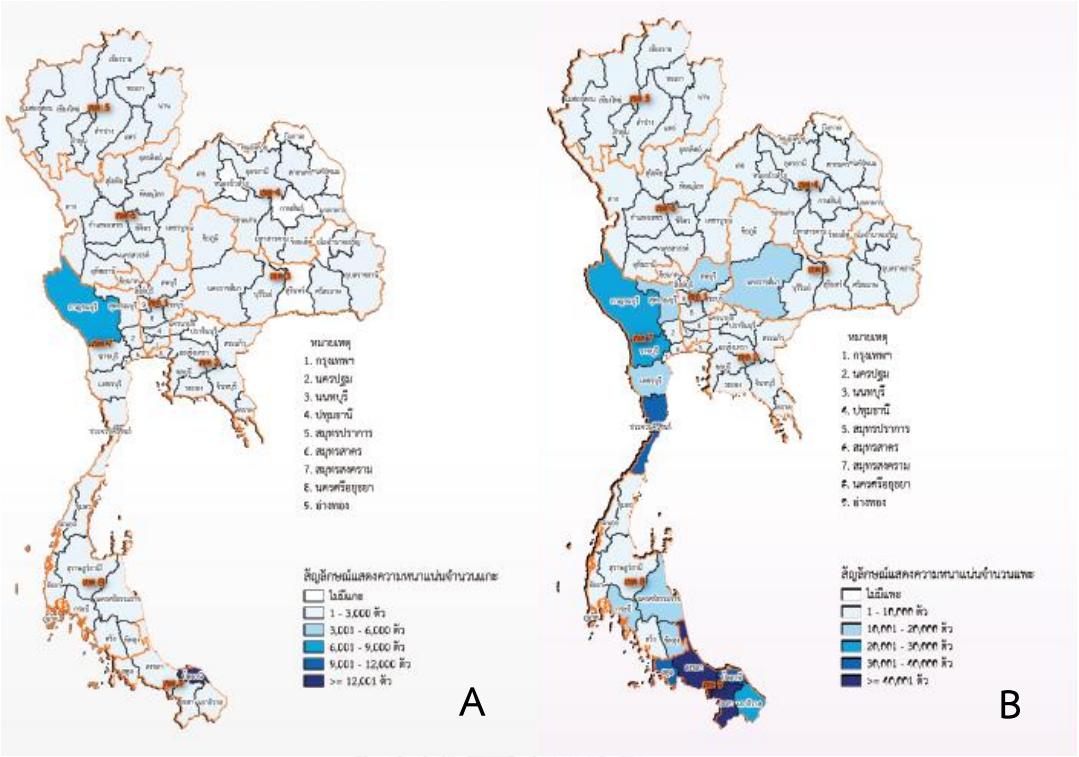


**Figure 1** Maps represented A) sheep and B) goat distributions (separated by livestock area and province) in Thailand (Source: Department of livestock development of Thailand)

The ART potentially accelerates the development of animal reproduction and therefore this technology has a great advantage for agriculture industry (D'Alessandro et al., 2001). The techniques include artificial insemination (AI), estrus synchronization, ovum pick-up (OPU), superovulation, *in vitro* production (IVP), embryo transfer (ET), embryo cryopreservation, cloning and transgenesis. Although these techniques have been intensively studied in cattle in Thailand, but the information for small ruminants is still limited. The ART in sheep are usually performed in several well-developed countries, optimization and novel knowledge would also be important for development of small ruminant industry in Thailand. Thus, overall objective of this thesis is to optimize the ART for genetic and reproduction improvement in small ruminants in Thailand.

To address this, several studies were performed. Firstly, to produce a high genetic crossbreed with disease-free animals by using artificial insemination (AI), a

technique of choice for male reproduction. Good selection of disease-free rams produces a number of offspring from inseminated females that can be more powerful than that of natural breeding, in particular to avoid inbreeding. It can also reduce the cost of ram management compared with natural mating. AI principally allows the rapid production of offspring from good genetic resources but application of this technology to the industry is fairly limited. AI technique combined with semen cryopreservation prevents the spread of venereal or contagious diseases, such as brucellosis and Q fever (Cognie, 1999). It also increases the variety of genetics in farm animals. However, semen cryopreservation process may be adversely affected to biochemical properties and functions of cryopreserved sperm, such as motility, viability, fertilizing ability, and all of which has an enormous impact on pregnancy rate (Labbe et al., 2001a). Therefore, optimal semen freezing medium that is beneficial for frozen-thawed quality of sheep spermatozoa needed to be studied.

Therefore, the fertility rates following laparoscopic artificial insemination (LAI) in small ruminants have generally been higher than other AI techniques (vaginal or cervical AI technique) (Gourley and Riese, 1990; Anel et al., 2005; Fair et al., 2005). The LAI is minimally-invasive surgical AI technique which is performed through two small incisions by well-trained veterinarian. This LAI technique allows directly deposition of the semen into the uterus. Although successful rate of sperm cryopreservation has been variable among the studies (Ritar et al., 1987; Moses et al., 1997; Sohnrey and Holtz, 2005; Martinez-Rojero et al., 2007), this technique reduces the numbers of inseminated spermatozoa in sheep and goats. Recently, Anakkul et al. (2013a) reported that this LAI was successfully performed in goat with a high pregnancy rate. LAI is therefore of interest to compare in sheep as well. Thus, our first objective is to apply LAI combined with frozen thawed semen to produce disease-free and selected genetically animals.

Secondly, embryo transfer (ET) is a technique of choices for dam-line selection. ET can be combined with AI to accelerate the genetic progress In ET process, the superovulation by administration of exogenous gonadotropin is frequently performed in order to increase the ovulations. The ovulated oocytes can then be fertilized and developed to embryos which can be collected and transferred

to several recipients. Classically, exogenous gonadotropins, such as follicular stimulating hormone (FSH) and equine chorionic gonadotropin (eCG), former name PMSG (pregnant mare serum gonadotropin) are frequently used in practice. However, eCG is less popular due to its long half-life, and it showed the increased proportion of unfertilized embryos, decreased embryo recovery rate, and embryos quality (Armstrong and Evans, 1983; Mobini et al., 2002). While FSH treated does produced more good quality embryos, however a multiple injection of FSH has to be performed due to its short half-life, then this result in extensive management, increased animal stress and time consuming. A single injection of FSH combined with slow releasing delivery systems has been proposed in bovine to reduce the above handicap. This fashion has been reported to give similar ovarian responses as multiple injection regimens (Lopez-Sebastian et al., 1993; Bo et al., 1994; Dattena et al., 1994; Sugano and Shinogi, 1999; D'Alessandro et al., 2001; Kimura et al., 2007). It was more applicable if superovulatory regimen was simpler using the slow release FSH regimen with hyaluronan. Therefore, our second objective is to compare the ovulatory stimulation regimens between single and multiple injections of FSH, in terms of ovarian responses and number of transferable embryos.

Thirdly, after acquiring a suitable and practical program, embryos product by the first and second topics were transferred to local bred ewes. Embryo transfer (ET) advances the genetic selection and reduces risk of disease spread both infectious and non-infectious diseases, such as viral, bacterial, and Scrapie diseases (Thibier and Guérin, 2000). The factors for successful ET consisted of donor and recipient management, estrus synchronization, superovulation program, insemination management, embryo collection, embryo evaluation, and embryo transfer process (Ishwar and Memon, 1996). However, information on successful transfer of embryos is fairly limited in Thailand.

In conclusion, the contents of this thesis work aim to study on semen cryopreservation, LAI, superovulation program, and embryo transfer in sheep and to apply in commercial scale to prove that these technologies can support the genetic requirement of the farmers in order to translate the technologies from the laboratory

to the farm. This is also important to Thailand whose the technology can be accepted and developed in laboratory scale successfully.


**Figure 2** Imported sheep in sheep farms in Thailand: Meat breed (Black Dorper, A), Dual-purpose breed (Black Sufflok, B; Cheviot, C; Black Romney, D) and Wool breed (Drysdale, E; Corriedale, F)

## 1.2 Literature reviews

### 1.2.1 Semen collection and cryopreservation in ram

Semen cryopreservation helps farmers not only selecting the high genetic merit ram but also reducing endemic spreading of infectious diseases especially those transmitted from ram semen. Successful development of cryopreservation and artificial insemination would allow achieving the pregnancy as similar to that of

natural breeding. Several techniques have been used to collect the semen from rams. The ram can also be trained for semen collection using among dummy, estrus ewe or non-estrus ewe. Among the techniques used for semen collection, artificial vagina (AV) is one of the favorite techniques because it is less invasive than an electro-ejaculation (EE) technique. Semen collection via artificial vagina yields good quality and quantity of the collected semen. EE is a technique using an electrical stimulatory probe. The probe (10 – 15 cm) will be inserted into the rectum adjacent to the accessory sex glands. This technique obtains greater semen volume compared with the AV technique but its result frequently associates with low sperm concentration (Moore, 1985; Jainudeen et al., 2000). Furthermore, EE would ethically need general anesthesia, so this technique is rather impractical for farmers as it needs to be performed under supervision of licensed veterinarian.

For semen cryopreservation, selecting a suitable freezing extender is the key factor in highly post-thawed semen quality. In generally, the semen cryoperserved extender is composed of a lot of nutrient and protective agent for sperm, i.e. Tris-(hydroxymethyl)-aminomethane, critric acid, cryoprotective agent (CPA), saccharides (sugars), and antibiotics (Salamon and Maxwell, 2000). CPA is divided into two types as penetrating CPA and non-penetrating CPA (Evans et al., 1987). Penetrating CPA, such as 4 – 8 % glycerol is often used for freezing ram and buck semen (Salamon and Maxwell, 2000; Aboagla and Terada, 2003; Ali Al Ahmad et al., 2008; Farshad et al., 2009). Glycerol is the most frequently used as sperm protective agent in freezing ram semen medium because it has been demonstrated to be better than other penetrating CPA including dimethylsulfoxide (DMSO), ethylene glycol, albumin, low molecular weight polyols, polymeric compounds, surfactants, high concentrations of sugar, compatible solutes (proline, glycine betaine and taurine) and antifreeze proteins from polar fish (Salamon and Maxwell, 1995). Non-penetrating CPA also plays a role in protecting sperm during cryopreservation because it can preserve sperm membrane property during freezing and thawing. Either egg yolk (EY) or skim milk is favorite for non-penetrating CPA in animal and human semen cryopreservation. Many studies reported that viability and motility rates of cryopreserved sperm with EY or skim milk were better than without

supplementation (Lardy and Phillips, 1939; Phillips and Lardy, 1940; Salisbury et al., 1941; Willett and Salisbury, 1942; Bergeron and Manjunath, 2006). The ranges of EY concentration can be variable from 15 – 50 percentages (v/v) for ram semen freezing extender (Abdelhakeam et al., 1991; Salamon and Maxwell, 2000). In addition, other supplements, such as sodium dodecyl sulphate (SDS) (Aboagla and Terada, 2003) or Equex STM® paste (Akourki et al., 2004; Anakkul et al., 2011) have been added into freezing extenders in order to improve semen quality during cryopreservation process. Saccharides are divided into monosaccharide, disaccharide and trisaccharide types. Each type has different properties to protect sperm during freezing and thawing (Medeiros et al., 2002). Appropriate sugar concentrations have been reported to be between 30 – 210 millimolar (mM). Farshad et al. (2009) presented that supplementation of combined two different types of sugar in semen cryopreserved extender improved sperm quality when compared with that of single sugar. These above mentioned results are in an agreement to the findings that combination of sugars better preserved buck sperm function, viability, and quality following cryopreservation (Naing et al., 2010). However, such study on interaction of sugars added into the semen extender and *in vitro*- and *in vivo*- post-thawed sperm quality has yet to be performed in rams. It is therefore of interest to study the effect of combination of sugars in order to increase ram sperm quality.



1.2.2 Reproductive cycle and synchronization in sheep

The ewe is seasonally polyestrus animal. The estrus cycle is usually 16 to 17 days with estrus period approximately 20 to 36 hours. The estrus can be classified to four stages including proestrus, estrus, metestus and diestrus. The estrus pattern in ewes is similar to goats but the ewe has shortened estrus cycle than the goat (Jainudeen et al., 2000). In the ewe, resting pools of primordial follicles (containing primary oocytes) are established prenatally. Three to four follicles recruited from the follicular pool and are committed to a growing pool (primary follicles) every day. FSH receptors express in granulosa cells quite early in the follicular development. At this stage, folliculogenesis appears to be gonadotropin independent during the one- to

two- millimeter stage. Follicles, except dominant follicle, degenerate and undergo atresia by a process of programmed cell death (apoptosis) (Scaramuzzi et al., 1993). In a cyclic ewe, the day of emergence of each follicular wave is synchronized to a small peak in FSH serum concentrations. Serum concentrations of estradiol increase from follicle wave emergence and peak when the dominant follicle develops. Termination of follicle development and decreased secretion of estradiol could provide a signal for the subsequent FSH peak that precedes and may induce the new wave of follicle emergence (Bartlewski et al., 1999a). During the estrus cycle, antral follicles emerge or grow from the pool of one-to two-millimeter diameter follicles, approximately every four days. The ovulatory follicle emerges around day 12 of the estrus cycle (Evans et al., 2000). Ovulation time in the ewe is between the middle of estrus intervals. After ovulation, the ovulated follicle contains a blood clot with few luteal tissues. It grows rapidly and develops to a corpus hemorrhagicum until day 4 or 5 of the cycle (ovulation = day 0). Progesterone hormone is produced by luteal cells when it develops to corpus luteum. Bartlewski et al. (1999b) found that numbers of luteal cells increased until day 10 after estrus, and then declined to day 17 of the estrus cycle; however, numbers of luteal cells did not significantly differ between days 8 and 13. During day 14 – 17 of the cycle, prostaglandin $F_2\alpha$ ($PGF_2\alpha$) produced by the endometrium of the uterine horn ipsilaterally to the corpus luteum causes luteolysis.



The control of estrus cycle or estrous synchronization in small ruminant can be performed using several hormonal regimens but there are two traditional programs, progestagen and prostaglandin programs. Progestagen or exogenous progesterone blocks FSH release. Several types of progestagen, such as fluorogestone acetate (FGA) and methyl- acetoxy progesterone (MAP) covered in sponge for vagina insertion have been used (Rathbone et al., 1998). In addition Norgestomet or Syncromate-B® for subcutaneous implant of progesterone at the back of ear skin, Controlled Internal Drug-Releasing device (CIDR®) for vagina insertion and oral progestagens have also been used to synchronize the estrous cycle. Exogenous progesterone is commonly used to synchronize breeding programs due to an effective means of controlling estrus and ovulation. Estrus synchronization with

progestins inserted 10 – 16 days period was successfully performed (Lunstra and Christenson, 1981; Killian et al., 1985; Jabbar et al., 1994; Stellflug et al., 1994). These programs are being used in conjunction with or without equine chorionic gonadotropin (eCG). The use of eCG plus progestin protocols in the ewe increases ovarian response, conception rate, and twin births from the induced estrus (Boscos et al., 2002). However, after repeated synchronization with eCG, the ewe presented poor estrus synchronization and reduced fertility or pregnancy rate probably because of eCG antibody formation (Bodin et al., 1997; Roy et al., 1999). Rubianes et al. (1999) presented that using $PGF_{2\alpha}$ at CIDR removal is effective up to 96 % synchronization with 70 – 80% pregnancy rate.

1.2.3 Laparoscopic artificial insemination (LAI) and insemination time in sheep

Artificial insemination in sheep can be performed via several techniques which are different in equipment, dose of insemination and site of sperm deposit. For vaginal artificial insemination, semen is usually placed in the vagina using an insemination pipette. The second technique for AI so called cervical artificial insemination involves the insert of a long tube aided by a vaginal speculum to pass through the cervix. The success of this technique however depends on depth of insemination in cervix and spermatozoan viability (Salamon and Maxwell, 2000). The intrauterine artificial insemination is an important technique for increasing fertility rate following AI with frozen-thawed semen in small ruminants. This technique can be performed using either transcervical or laparoscopic intrauterine insemination. However, transcervical artificial insemination in the ewe is complicated and difficult because of the unique of cervical anatomy (small cervix with many cervical folds). Therefore, laparoscopic artificial insemination is a good choice for successful insemination in the ewe. It is a minimally invasive surgical AI that allows the deposition of semen into the uterine lumen. This technique yields higher successful rate than other methods because sperm deposit site is close to the ovulation area (Gourley and Riese, 1990; Anel et al., 2005; Fair et al., 2005).

Many studies reported that the results of fertility rates in the ewe and doe after laparoscopic AI with cryopreserved semen were ranged between 33.3 – 71.2 % in goats (Ritar et al., 1987; Sohnrey and Holtz, 2005; Martinez-Rojero et al., 2007) and 40.5 – 98.2 % in sheep (Eppleston and Roberts, 1986; Casares et al., 1990; Gourley and Riese, 1990; Halbert et al., 1990; Findlater et al., 1991; Gimenez-Diaz et al., 2012). Recently, in Thailand, Anakkul et al. (2014) reported highly success rate (about 60%), even though the semen was deposited in opposite site of ovulation. Moreover, a successful insemination is still related the timing of insemination and ovulation. Higher fertility or pregnancy rates can be achieved when insemination proceeded between 48 and 72 hours after progestagen removal (Maxwell, 1986; Findlater et al., 1991). Maxwell (1986) found that the average time of ovulation occurred at around 55.8 hours after sponge removal.

1.2.4 Superovulation in sheep

Superovulation or multiple-ovulation induction is a hormonal technology to promote follicle development and to increase follicle numbers by exogenous injections of follicle stimulating hormone (FSH) or FSH-like hormone (such as equine chorionic gonadotropin, eCG). This technique mimics the pulsatile secretions of FSH from anterior pituitary glands. Classically, exogenous FSH or FSH-like hormone will need to be administrated during late diestrus prior to the selection of a dominant follicle. This result in an increase of the numbers of follicles can be used for oocyte collection via ovum pick-up or for embryo collection after insemination. The simplest method for superovulation is to give 500 – 1,500 IU pregnant mare serum gonadotrophin (PMSG) or eCG. Because its long half-life of approximately 72 hours, single injection of eCG is sufficient to stimulate the follicle growth. However, it acts as LH-like rather than FSH-like activities and also produces a larger number of unfertilized ova and poor quality embryos, stimulates PMSG antibodies, abnormally estrus cycle, abortion and also cause prematurely CL degeneration (Amoah and Gelaye, 1990). At present, FSH has surpassed eCG as the program of choice for superovulating animals. In comparison of the two types of hormone, the FSH

treatment resulted in slightly higher yield of transferable embryos than that obtained from eCG (Kiessling et al., 1986; Amoah and Gelaye, 1990; Goel and Agrawal, 1990; Jabbour and Evans, 1991; Mahmood et al., 1991; Nowshari et al., 1992; Pendleton et al., 1992; Pintado et al., 1998; Goel and Agrawal, 2005). Greyling et al. (2002) reported that the follicle response and number of embryos with FSH stimulation in small ruminant depend on donor ages, season, temperature, body score, health, number of parity and animal breeds. In practice, since the FSH has short half-life *in vivo*, the injection of FSH usually performed two times a day for three to five days with decreasing doses in order to maintain serum FSH levels. The multiple injections of FSH are then, stressful for animals, labor intensive, and time consuming (Dattena et al., 1994; D'Alessandro et al., 2001). The stress from repeatedly FSH injections can affect follicle development response (Bo et al., 1994). Therefore, novel strategy to minimize the animal interventions and injections such as slow FSH release technology would be required.

1.2.5 Superovulation with a single injection of FSH

A single FSH injection seem advantageous in reduction the stress from multiple handlings of animal and less time consumption. Recently, challenges have been made to improve multiple-ovulation programs with reducing the numbers of FSH injection. A single superovulation injection with a biodegradable polymer in ruminant have been studied by several researchers (Lopez-Sebastian et al., 1993; Yamamoto et al., 1995; Satoh et al., 1996; Sugano and Shinogi, 1999; D'Alessandro et al., 2001; Kimura et al., 2007; Tríbulo et al., 2012). Several biological polymers have been applied to sustain hormone release in animals. In bovine, administration of FSH dissolved in various substances, for example, polyvinylpyrrolidone (PVP), aluminum hydroxide gel and hyaluronan, resulted in an increase ovarian response when compared with FSH multiple injections (Bo et al., 1994; Yamamoto et al., 1994; Yamamoto et al., 1995; Kimura et al., 2007; Bó et al., 2009; Tríbulo et al., 2011; Tríbulo et al., 2012). In Thailand, Chasombat et al. (2013) demonstrated that single

injection with FSH combined with PVP significantly increased the numbers of ovulatory follicles compared to multiple FSH injections in Thai native heifers.

Hyaluronan so-called hyaluronic acid or hyaluronate (HA), is an anionic-nonsulfated glycosaminoglycan that functions as major component of extracellular matrix (figure 3). HA has been used in many medical applications, such as corneal transplantation, osteoarthritis treatment, induces tissue healing, and cosmetic. Tríbulo et al. (2012) showed in cattle that FSH dissolved with hyaluronan and injected singly, tended to increase the numbers of fertilized and transferrable embryos than that of control group (multiple injection of FSH in saline). However, no study on the effects of single FSH injection with hyaluronan on superovulatory response in ewes has yet to be examined. In present thesis, superovulatory effect of single FSH injection combined with slow release hyaluronan in sheep will be elaborated.

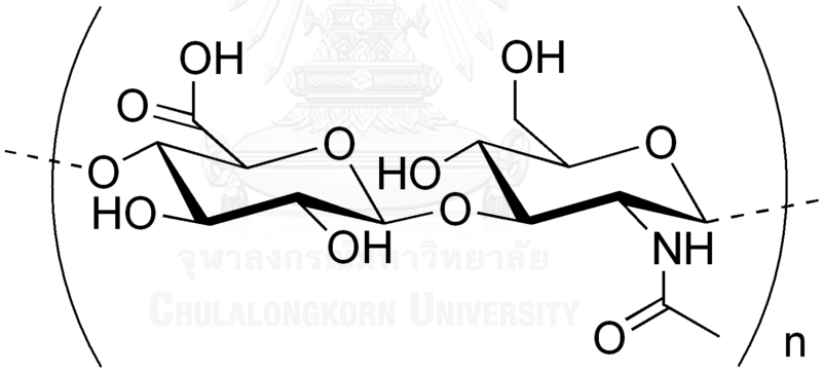


**Figure 3** Structure of hyaluronan which is a polymer of disaccharide, composing of D-glucuronic acid D- N- acetylglucosamine (modified from https://en.wikipedia.org/wiki/Hyaluronic_acid)

1.2.6 Embryo transfer in sheep

Embryo transfer is an important technology for rapid lamb production in highly genetic breeds. It reduces lamb production interval, helps widespread distribution of the superior genetic, and controls risk of spreading diseases in sheep flock including viral, bacterial, and Scrapie diseases (Thibier and Guérin, 2000). Nowadays, traditional ET technology in bovine is performed by non-surgical embryo

transfer but not yet to develop in ovine. ET in small ruminants can be reliably succeeded by laparoscopic procedure (McKelvey et al., 1985; McMillan and Hall, 1994). Laparoscopic ET is more safely and minimally invasive surgery than laparotomy. This technique is less time consuming for ET and the results for conception rates have been reported to be similar to mid-ventral laparotomy ET (Cognie, 1999). Factors affecting survival of transferable embryos are site of transfer, numbers of embryo at transfer, and the synchrony between embryo developmental stage and stage of recipient. Previous studies concluded that an early stage embryo (2- to 16- cells embryo) should be transferred into the oviduct, but the morula or blastocyst stages will need to transfer into the uterus at ipsilateral site of observed ovulation (Averill and Rowson, 1958; Moore, 1974; Armstrong and Evans, 1983). High pregnancy rate after ET improved when two embryos were transferred compared with one embryo transfer (Armstrong et al., 1983; Tervit et al., 1986). Armstrong et al. (1983) found that the incidence of twin birth was highly significant when unilateral transfer with two embryos than bilateral transfer. The collected embryos from donor should be transferred to estrus-synchronized recipient before 12 hour to after 12 hour of their respective donors (Moore and Shelton, 1964). In Thailand, Anakkul et al. (2013a) successfully produced new breed line of black goats using ET program.



**1.3 Objectives of the thesis**

1. To examine the effects of different types of sugar supplemented into freezing medium on post-thawed quality of ram semen.
2. To determine the fertility of ewes after laparoscopic artificial insemination using cryopreserved ram semen with sugar combination in freezing medium.
3. To assess the ovulation rates and number of embryos in superovulation program with single injection of FSH dissolved with hyaluronan compared with multiple injections of FSH.
4. To determine the fertility of ewes after embryo transfer produced with single or multiple injections of FSH in sheep.

**1.4 Hypothesis**

1. Supplementation of different sugar into freezing medium affects post-thawed quality of ram sperm.
2. Fertility after laparoscopic artificial insemination using cryopreserved ram semen with sugar combination in freezing medium is equal to fresh semen.
3. Single injection of FSH dissolved with hyaluronan in superovulation regimen improves ovarian response and transferable embryos in ewes.
4. Fertility after embryo transfer using embryos produced with single injection of FSH dissolved with hyaluronan is similar to embryos produced with multiple injections of FSH (traditional superovulation program).

**1.5 Keywords:** Embryo transfer (ET), Hyaluronan, Laparoscopic artificial insemination (LAI), Semen cryopreservation, Sheep, Single injection of FSH for superovulation

**1.6 Research merits**

1. The appropriate sugar supplementation in semen freezing extender for ram semen cryopreservation.
2. The effective and minimal stressful superovulation program with single injection of FSH in ewes under FSH dissolved with hyaluronan.
3. The knowledge about embryo quality between two differences of *in vivo* embryo producing programs (single v multiple supreovuation) in the sense of fertility after embryo transfer.
4. The applicative protocol of artificial insemination with post-thawed semen or/and embryo transfer for produce offspring lamb in sheep industry.
5. National and international publications

# CHAPTER II

# HEAT STRESS CAN AFFECT SEMEN PRODUCTION IN IMPORTED RAM: A CASE REPORT

## 2.1 Abstract

The aim of this study was to investigate the effect of seasonal and environmental conditions on ejaculated semen qualities and male reproductive traits in an imported ram under tropical conditions. A Dorper ram was imported from the South Africa and raised in Nakhon-Pathom province. The results showed that this ram's ejaculated semen characteristics (volume, concentration, mass motility, motility, and percentage of normal spermatozoa) and scrotal circumference varied according to the temperature-humidity index (THI) and day length among months. The characteristic peak of the Dorper ram's reproductive traits was during the period from December to March.

## 2.2 Introduction

The production of high semen quality from a high genetic potential male is a key to success in breeding improvement through crossbreeding. Therefore, the importation of male animals of high genetic merit is a practical action to improve reproduction and expand an animal herd. One key factor that affects semen production in males is heat stress (Hansen, 2009). In tropical countries, poor reproductive performances, including male sexual behavior, semen production, and quality, have been seen to decline (Kumi-Diaka et al., 1981; Ahmed et al., 1997; Suriyasomboon et al., 2005; Nichi et al., 2006; Koonjaenak et al., 2007). In bovine raised in a tropical region, the extended period of humid-temperatures and longer day lengths was found to affect reproduction (Bouraoui et al., 2002; Somparn et al., 2004; Suadsong et al., 2008); this was also found in swine reproduction (Kunavongkrit

and Heard, 2000), as semen volume and quality reduced significantly in summer. It has also been found that a high temperature and high relative humidity have a negative effect on goat's reproduction as these conditions reduce semen volume and sperm concentration (Mukherjee et al., 1953; Hiroe and Tomizuka, 1966; Yokoki and Ogasa, 1977). However under tropical conditions with high ambient temperature and humidity, semen production of rams is still unclear. Therefore, this research studied one case of a Dorper ram imported from South Africa to be a breeder to determine if male reproductive performance was affected by climatic conditions in Thailand.

## 2.3 Materials and Methods

### 2.3.1 Animal

A Dorper ram born in the South African in September 2011 was purchased and brought to the University Large Animal Hospital in Nakhon-Pathom province (Central Thailand) in October 2012. After arrival, the ram appeared physically normal and received a general health examination, including physical, complete blood count (CBC), and fecal examination, and was tested for brucellosis, bluetongue, and paratuberculosis. It was then housed in a semi-intensive protective system and clinical veterinary care. It was fed individually with concentrate (12% crude protein) and grass ad libitum. Drinking water and mineral block were made available at all times.

### 2.3.2 Semen collection and evaluation

The scrotal circumference was measured monthly around the testicular scrotum with the greatest width using a tape measure. Semen was collected using an artificial vagina (AV) technique as described by Moore (1985). The semen was collected once a month beginning 4 weeks after the ram was moved to the location. Semen volume, consistency, and color were macroscopically evaluated at instant post-ejaculation time. Semen volume was assessed from a graduated collecting tube.

The color and consistency of the evaluated semen was classified as creamy, milky, opaque, or watery.

The pH of semen was measured with a pH-indicator paper (Neutralit®, Merck, Germany). Mass motility and motility of ejaculated semen were evaluated under a phase contrast microscope at 40X and 100X magnification, respectively (Olympus® microscope model, Shinjuku, Japan), using the numerical scale 0 – 4 mass motility (Islam et al., 2006). The sperm concentration was diluted 1: 400 and determined by a hemocytometer (Neuber®, Town, Germany). Stained semen smear was performed by diluting mixed semen with eosin-aniline blue stains to examine sperm morphology.

Climatic data (temperature, relative humidity, and day length) were provided by the Meteorological Department of the Ministry of Communications of Thailand. Data were collected from meteorological stations in Nakhon-Pathom, Thailand for period of one year, from July 2013 to June 2014. Data were collected at 4:00 am and 4:00 pm to represent morning and evening climates.

The THI was calculated at each time point using the equation as developed by Spiers et al. (2004), a dimensionless static, which is expressed as:

$$THI = (((1.8 \times T) + 32) - (0.55 - (0.0055 \times H))) \times (((1.8 \times T) + 32) - 58)$$

where $T$ is the mean value of temperature in °C and $H$ is the % of mean value of relative humidity.



**2.4 Result and Discussion**

The variation of temperature, day length and THI at the experiment site are shown in Fig 4. It was found that testicular size was significantly larger in months with lowest temperature, from December to March, when compared to other months (mean testicular 36.8 v 32.2 cm, respectively. Heat stress had a significant effect on semen quality, as while the animal still showed a libido, watery ejaculated semen was collected, but no sperm was found. Due to the fact that a complete duration of a ram's spermatogenic cycle is 47 – 48 day (Zeng et al., 2006), the heat stress does seem to affect semen quality in the next 1.5 – 2 months. This observation corresponds to Irahim (1997), who reported that the highest peak temperature during

summer had a negative effect on semen quality in rams. Similarly, Karagiannidis et al. (2000), studying Chios and Friesian rams, reported that the positive correlation between high ambient temperatures and increasing day length during summer months caused a decrease in ejaculated semen quality. The semen quality of an imported ram did improve when the temperature started to decrease in October, and the animal was able to reproduce sperm of a higher quality beginning in December. In contrast with our findings in the imported Dorper ram, a higher quality semen for insemination or freezing was found in male goats as they appeared to adapt better to a hot climate when compared with sheep and cattle (Coop, 1982; Valez-Nauer et al., 1982). Although, seasonal conditions had a pronounced effect upon ejaculated semen traits in goats, an acceptable quality semen is still reproduced throughout the year (Anakkul, 2012).

THI is an indicator used to measure the effect of heat stress on ruminant production in tropical or subtropical areas (Bouraoui et al., 2002). In this study's region, THI had mostly mild stress (72 – 78) in the morning and medium stress (79 – 88) in the afternoon (see also figure 4A). This study found that male reproductive performance (ejaculated semen quality and testicular size) improved when THI value decreased from 74 to 64 and 80 to 75 in am and pm, respectively. El-Darawany (1999) reported that rams suffered from severe heat stress when THI was above 84.3. In addition, the decrease in scrotal and testicular circumferences during high ambient temperature agree with Yarney et al. (1990). Their study found that scrotal circumference, testicular size and tone are great indicators of spermatogenesis and sperm production in rams.

**Table 1** Semen characteristics (semen volume, pH, total number of sperm, mass motility, % motility, % normal sperm morphology of head and tail) of imported ram in each month during 1 year (July 2013 – June 2014).

| Month | Semen volume (ml) | pH | Total number of sperm ($\times 10^9$) | Mass motility[1] | Motility (%) | Normal head (%) | Normal tail (%) |
|---|---|---|---|---|---|---|---|
| **Jul-13** | 1.75 | 7.5 | 1.3 | 2 | 55 | 47.8 | 80.2 |
| **Aug-13** | 2 | 7 | 1.1 | 0 | 0 | 52.2 | 72 |
| **Sep-13** | 1.5 | 7.5 | NA | 0 | 0 | ND | ND |
| **Oct-13** | 1 | 8 | NA | 0 | 0 | ND | ND |
| **Nov-13** | 1.8 | 7 | NA | 0 | 0 | ND | ND |
| **Dec-13** | 2.5 | 7 | 1.8 | 3 | 85 | 99.4 | 95.4 |
| **Jan-14** | 3.8 | 7.5 | 6.5 | 3 | 70 | 99.2 | 88.5 |
| **Feb-14** | 3.5 | 7.5 | 1.8 | 2.5 | 65 | 98.8 | 85 |
| **Mar-14** | 1 | 7 | 1.5 | 3.5 | 70 | 98.4 | 87.5 |
| **Apr-14** | 2 | 7 | NA | 0 | 0 | ND | ND |
| **May-14** | 2.5 | 8 | 0.2 | 0 | 0 | 46 | 72 |
| **Jun-14** | 3 | 7.5 | 1.0 | 2 | 60 | 54.2 | 77.5 |

[1] Based on a scale 0 to 4; 4 = best

NA = Not applicable caused number of sperm less than 10 million

ND = Not detected

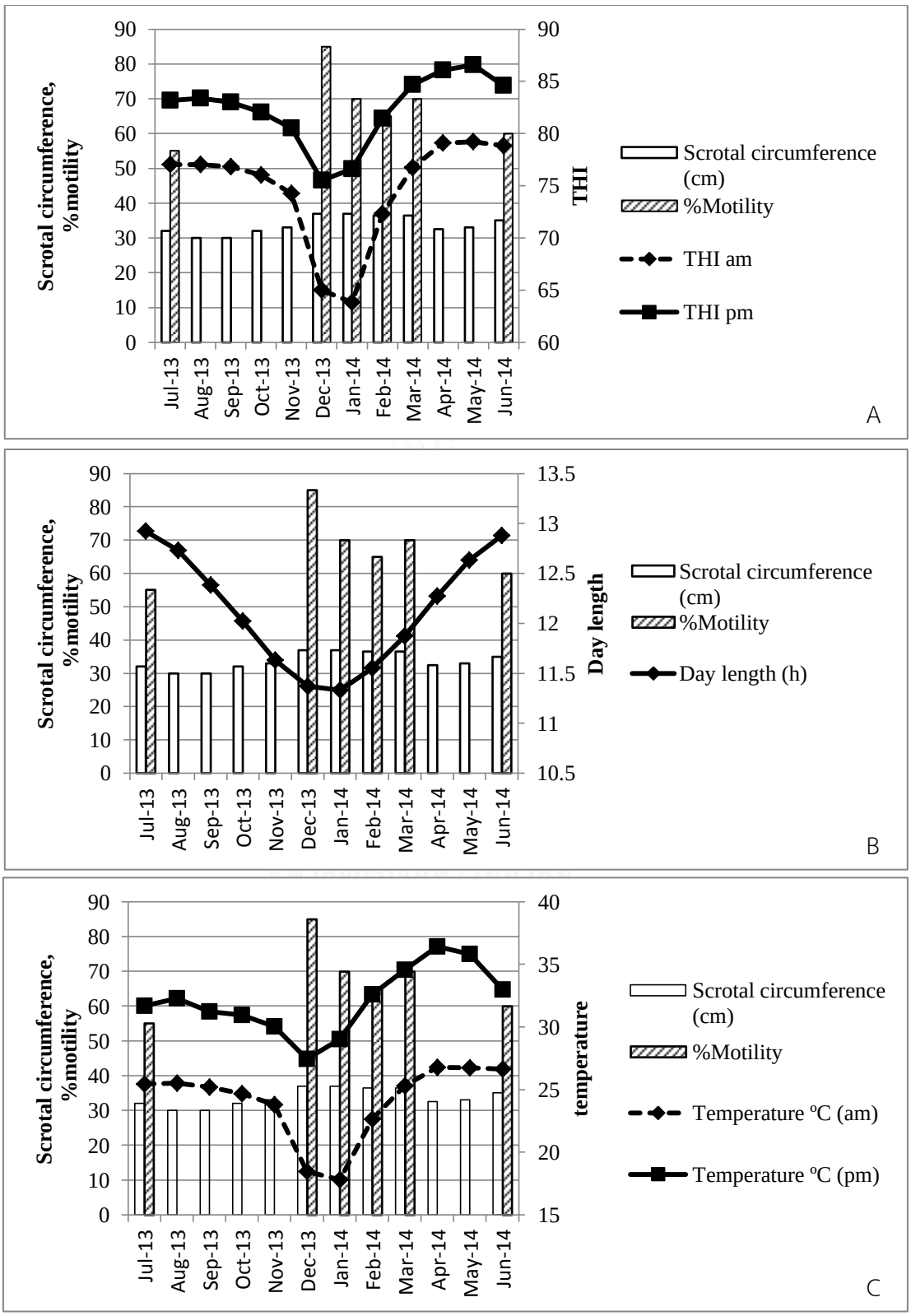


**Figure 4** Climatic data, percentage of sperm motility and testicular circumference of imported ram in each month during the study periods (July 2013 – June 2014).

Furthermore, the percentage of sperm abnormality increased with higher temperatures and humidity. The most common morphological abnormalities of sperm observed were tailless, microcephalic, spermatid, and coiled tail around the head (see also figure 5). Percentage of abnormal sperm morphology was higher during the non-breeding season (spring and summer), while the breeding season was a transferrable period with normal sperm (Mickelsen et al., 1981; Karagiannidis et al., 2000). It should be noted that semen production and sperm output recovered when the heat stress disappeared during the cool season. This means that semen production in an imported ram can recover if the climatic conditions return to normal range.

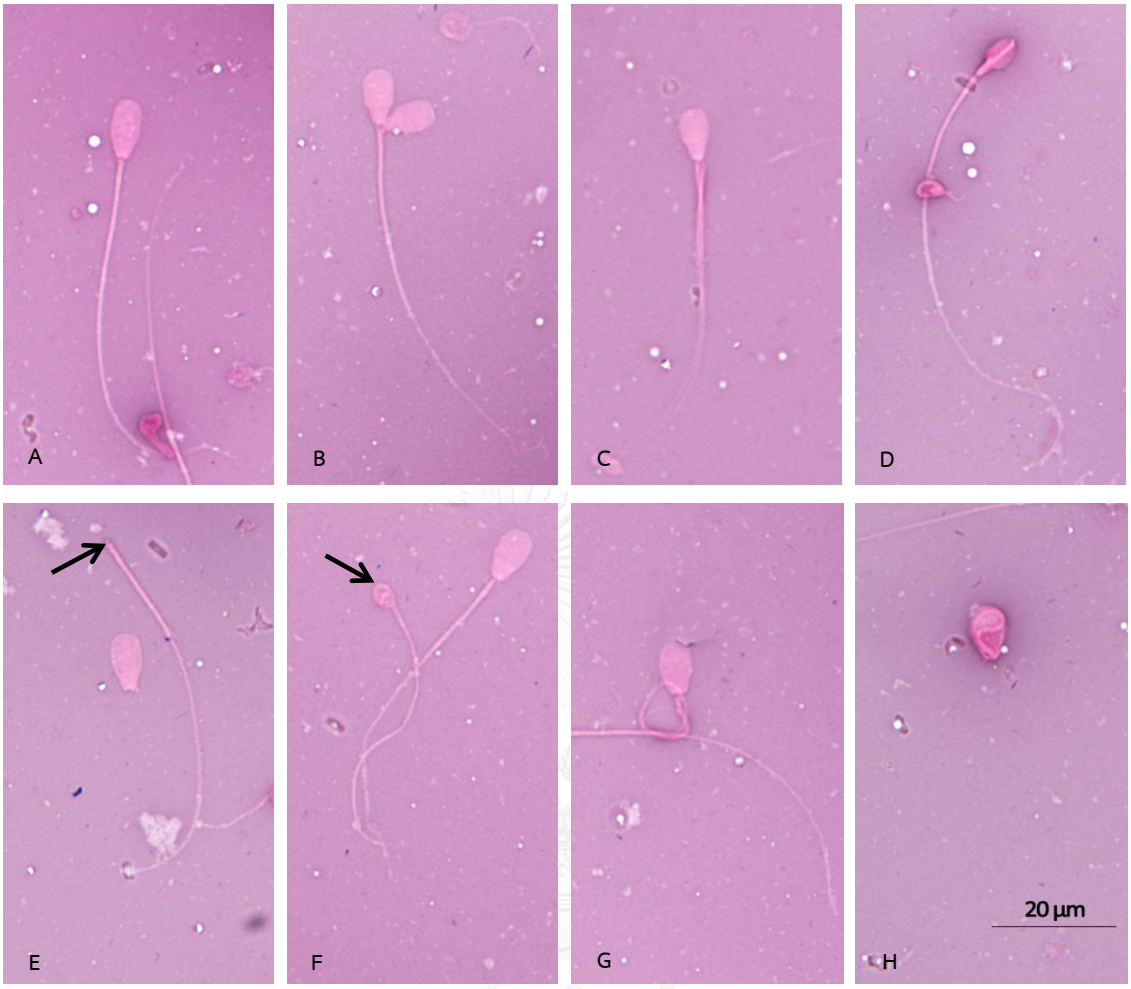


**Figure 5** Microscopic view of ram sperm stained with Eosin-Aniline blue stain (magnification: 1000X). Normal spermatozoa (A); Bicephalic (B); Biflagella (C); Microcephalic (D); Headless tail (arrow) and tailless head (E); Spermatid (arrow; F); Tightly coiled tail (G); and Coiled tail around the head spermatozoa (H).

In conclusion, this study's finding indicates that the reproductive performances semen traits and testicular size of a imported Dorper ram in Thailand varied according to seasonal conditions. The ram showed higher performances during the winter, cooler season (from December to March). However, good management strategies for decreasing heat stresses, for example, shading, a cooling system or air conditioning, can improve semen quality in a tropical climate.

# CHAPTER III

# EFFECT OF VARIOUS COMBINATIONS OF SUGAR SUPPLEMENTATION IN THE EXTENDER ON FROZEN-THAWED RAM SEMEN QUALITY AND FERTILITY

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## 3.1 Abstract

Semen cryopreservation alters sperm structure and biochemical properties, and subsequently impairs fertilizing ability. A combination of two different sugar molecules revealed an enhanced beneficial effect on frozen-thawed spermatozoa of various species. However, the report in ram has been scarce. The present study investigated the effect of differential combinations of sugar supplementation on post-thawed ram semen qualities in a cryopreservative extender in the presence of a single sugar or a combination of sugars. Data showed that a combination sucrose and trehalose significantly improved cryopreserved sperm motility, viability, longevity, and acrosome integrity. Fertility rates following laparoscopic artificial insemination using frozen-thawed semen (n = 35) with sucrose and trehalose supplementation revealed the same values as those utilizing fresh semen (n = 32) (82.9 v 84.4%, respectively). Our findings suggest that the use of a Tris-citric extender with the addition of sucrose combined with trehalose is an effective extender for ram semen cryopreservation. The optimization of concentrations and ratios between the combination of sucrose and trehalose supplemented in ram semen freezing extender should be further examined.

## 3.2 Introduction

Artificial insemination combined with semen cryopreservation is not only a technique of choice for ram industry, but it also prevents the spread of venereal or

contagious diseases (Cognie, 1999; Richardson et al., 2011). Furthermore, it expands a variety of genetics in farm animals. However, the cryopreservation technique has shown an detrimental effects on biochemical properties and functions of cryopreserved spermatozoa, i.e. motility, viability, and fertilizing ability, all of which have an enormous impact on pregnancy rate (Labbe et al., 2001b; Richardson et al., 2011).

Regarding semen cryopreservation, a freezing extender plays a central role in protection of spermatozoa toward cryoinjury, resulting in an enhanced post-thawed semen quality. The ram semen freezing extender is generally composed of Tris for acid-base buffer, citric acid for osmotic pressure balance, cryoprotective agent (CPA) for cryoinjury prevention during cryopreservation and most importantly, saccharides (sugars) for supporting energy and protection the spermatozoa (Abdelhakeam et al., 1991; Salamon and Maxwell, 2000; Paulenz et al., 2002; Mortimer and Maxwell, 2004; Câmara et al., 2011). Many studies reported the advanced effects of sugars in a semen freezing extender on the post-thaw viability of animal spermatozoa (Garcia and Graham, 1989; Molinia et al., 1994; Garde et al., 2008; Tonieto et al., 2010). Sugars are divided into monosaccharide, disaccharide, and trisaccharide types. Each type has different cryoprotective properties to protect spermatozoa during freezing and thawing (Yildiz et al., 2000; Medeiros et al., 2002).

Since monosaccharide is found mainly in the seminal plasma of ram. It is therefore a necessary supplementation for a semen freezing extender as an energy source and a protective agent for spermatozoa (Salamon and Maxwell, 2000), while disaccharide (sucrose or trehalose) provides effective protection for phospholipid membrane of sperm head, like CPA. However, recent studies suggested that post-thawed sperm qualities were improved by a semen freezing extender supplementation with a combination of mono- and disaccharides such as trehalose and sucrose in mammal (Aisen et al., 2002; Aboagla and Terada, 2003; Farshad and Akhondzadeh, 2008). Focusing on buck semen, Farshad et al. (2009) showed that an addition of sucrose plus trehalose to a freezing extender improved semen quality when compared with that of an individual sugar. Naing et al. (2010) also found that a combination of sugars supplemented in freezing extender provided an improvement

to buck sperm function, viability, and quality following cryopreservation. Few studies purposed that the use of a combination of fructose and trehalose (FT) achieved a higher percentage of viable and intact spermatozoa in ram (Aisen et al., 2000; Aisen et al., 2002; Matsuoka et al., 2006). To our best knowledge, the effects of various combinations of sugars in a freezing extender on post-thawed ram sperm qualities and fertility have never been studied. Thus, the present study aimed to determine the effects of single and combinations of sugars supplemented to a freezing extender on post-thawed quality of Dorper breed ram semen. A fertility test was further carried out by laparoscopic artificial insemination of cryopreserved semen with selected freezing extender in comparison with fresh semen.

## 3.3 Materials and Methods

All chemicals in the present study were purchased from Sigma-Aldrich Chemical Company (Sigma, St. Louis, MO, USA), unless stated otherwise.

### 3.3.1 Experiment 1 Effect of different sugar supplementation on post-thawed ram sperm quality

#### *3.3.1.1 Experimental animal*

The use of animals and research methodology of this study were approved by the Institutional Animal Care and Use Committee (IACUC), Chulalongkorn University (Approval No. 13310050). Three mature pure-bred Dorper rams (aged 2 to 3 years) were used as semen donors. They were housed in a semi-intensive system under preventive and clinical veterinary care, and fed on concentrate feed 0.4 kg/day/animal (12% protein) and *ad libitum* of grasses with free access to mineral blocks and water.

#### *3.3.1.2 Preparation of basic and testing semen extenders*

The basic semen freezing extender used in this study was Tris-citric acid (TC) solution (300 mM Tris, 95 mM citric acid), supplemented with 15% (v/v) hen's egg

yolk, 5% (v/v) glycerol (Sigma, Steinheim, Germany) (Mortimer and Maxwell, 2004), and 0.5% (w/v) Equex STM paste (Nova Chemical Sales Inc, Scituate, USA). Testing extenders were prepared by adding one or two types of sugar into the TC solution for a final concentration of 30 mM. pH and osmolality of the extenders were adjusted to 6.8 – 7.0 and 400 mOsm/kg, respectively.

*3.3.1.3 Semen collection and processing*

Semen was collected from the rams by an artificial vagina (AV) twice a week as described by Moore (1985) (Figure 6). Semen with acceptable motility (> 70%), sperm concentration (> 1,500 x $10^6$ spermatozoa/ml), and normal morphology (> 90%) was pooled and divided into aliquots. Each aliquot was diluted with one of the testing extenders. Concentration was adjusted to 320 × $10^6$ spermatozoa/ml. The diluted semen was equilibrated at 4°C for 4 h. After equilibration, it was loaded into 0.25 ml French mini straws (Minitüb®, Landshut, Germany). The straws were placed horizontally, approximately 4 cm above liquid nitrogen levels for 10 min in a Styrofoam box, before being plunged into liquid nitrogen for storage. After 24 h storage, one straw of each testing extender was randomly selected and thawed in a 37°C water bath for 30 sec (Salamon and Maxwell, 1995).


**Figure 6** Dorper ram as the semen donor in experiment (A), and ram semen was collected by artificial vagina (AV) technique (B).

*3.3.1.4 Assessment of sperm quality*

All semen was evaluated at five time points as follows: within 5 min (T0), 1 h (T1), 3 h (T3), 6 h (T6), and 9 h (T9) after thawing. The motility of post-thawed semen was evaluated by a computer-assisted sperm analyzer (CASA, Hamilton-Thorne Biosciences IVOS, Version 12.3, Bevery, MA, USA), at a setting recommended by the manufacturer. Assessment setup parameters were as follows: Frame rate, 60 Hz; cell size (min/max), 12/80 $\mu m^2$; and minimum curvilinear velocity (VCL), 10 /μm. Samples of frozen semen to be analyzed with CASA were thawed and warmed at 37°C. At least 500 sperm cells (6 fields) were selected and assessed for motility (Mo, %); average path velocity (VAP, μm/s) straight-line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); straightness (STR, %); and linearity (LIN, %). Sperm viability (Via) was evaluated using eosin-aniline blue staining. One drop of semen was mixed with eosin-aniline blue stain and thin-smeared onto a glass slide. The slide was then observed by a light microscope, and total count was 200 spermatozoa. Live spermatozoa, which were colorless, were classified as positive result, whereas dead spermatozoa, which were red, were classified as negative result.

A fluorescein isothiocyanated peanut agglutinin (FITC-PNA) was used to evaluate acrosome integrity (Acro) (Grasa et al., 2006). Thawed semen was diluted with 1: 5 phosphate buffered saline (PBS). Six μl of semen mixture was smeared onto a glass slide, and sperm membrane was permeabilized with 95% ethanol for 30 sec. The slide was covered with FITC-PNA (100 μg/ml in PBS) solution and incubated in a moist chamber at 4°C for 30 min, then rinsed with 4°C distilled water before air drying. The stained slides were evaluated using an epifluorescent microscope (BX51; Olympus®, Shinjuku, Japan). At least 200 spermatozoa per sample were evaluated. A spermatozoon with intense bright green fluorescent of acrosomal cap was indicated as intact acrosome (Figure 7).

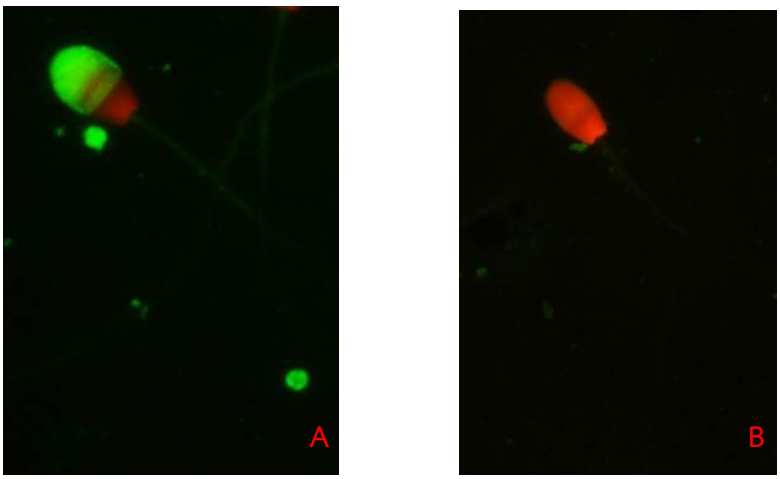


**Figure 7** FIT C-PNA fluorescence staining for evaluate acrosomal sperm integrity in ram semen. Spermatozoa with (A) or without (B) bright green stained over the head were classified as acrosome intact or reacted sperm**,** respectively.

3.3.2 Experiment 2 Effect of sugar supplementation in extenders used in fresh and frozen semen on fertility rate after laparoscopic artificial insemination (LAI)

*3.3.2.1 Experimental animals*

A total of sixty-seven multiparous local-bred ewes between 2 to 4 years of age were selected and randomly divided into two groups: fresh and frozen semen groups. The animals were managed the same as the ram semen donor group, but were fed with 0.3 kg/day/animal concentrates containing at least 14% (w/w) protein and water ad libitum.

*3.3.2.2 Estrus synchronization*

Estrus-synchronization was performed by intravaginal progesterone implant, (CIDR-G® ,Eazi-Breed™, 0.3 g progesterone in silicone, Interag, Hamilton, New Zealand) for 10 days, with an injection of 300 IU eCG (Folligon®, Intervet Schering – Plough Animal Health, The Netherlands) at the time of progesterone removal. Standing estrus was detected by a teaser ram 2 times/day (am/pm). Laparoscopic artificial insemination was performed at 22 – 24 h after standing estrus.

*3.3.2.3 Semen preparation process for artificial insemination*

Frozen-thawed semen was selected from the best quality semen in experiment 1.2. In the fresh semen group, the ejaculated Dorper ram semen was diluted to 320 × $10^6$ spermatozoa/ml, the same as the freezing semen extender but without glycerol and Equex STM® paste.

*3.3.2.4 Laparoscopic artificial insemination*

All ewes submitted to the operation were restricted from feed for 24 h and water for 12 h. They were anesthetized with xylazine HCl (0.025 mg/kg) combined with ketamine HCl (1.1 mg/kg) and given 0.04 mg/kg phenylbutazone as an analgesic. The LAI technique followed the procedure described by Ehling et al. (2003) and Anakkul et al. (2013a) (shown as Figure 8A). The greater curvature of each uterine horn was pierced with an insemination needle and either fresh or frozen-thawed semen was deposited into the uterine lumen (Figure 8B). The insemination dose was 40 × $10^6$ spermatozoa/horn. The insemination was done under observation via a direct view 5-mm laparoscope (Schölly, Denzlingen, Germany) (Alfaris et al., 2012). Post-operative care included intramuscular injection of 20,000 IU/kg penicillin-streptomycin and wound dressing with antiseptic for 3 days.

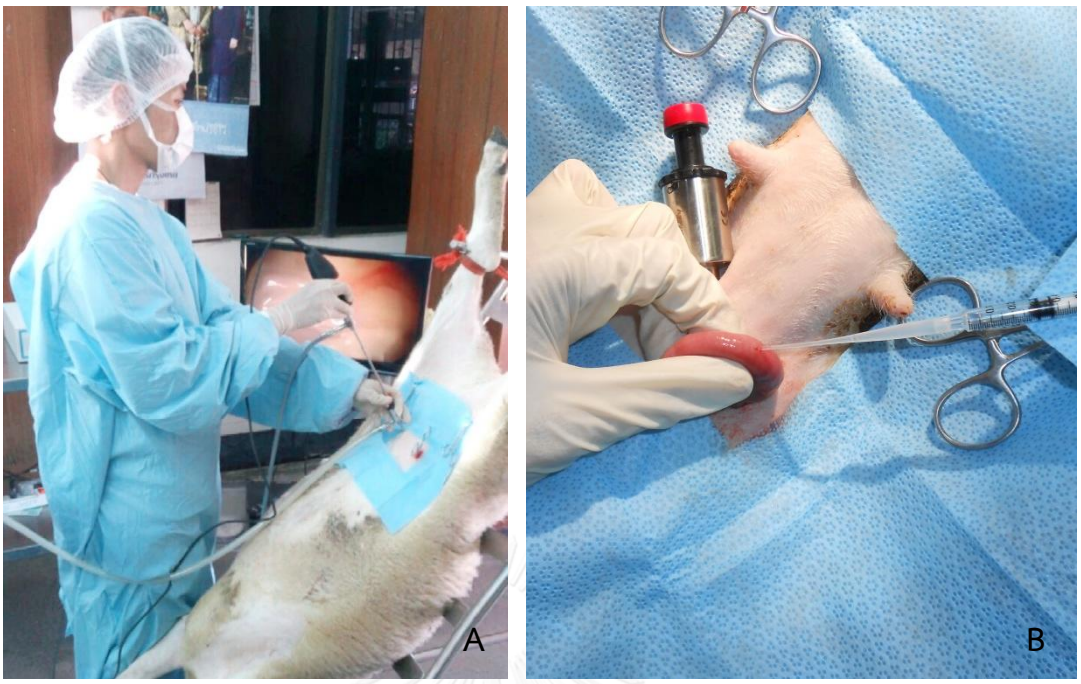


**Figure 8** Laparoscopic artificial insemination (LAI) procedure in sheep (A). The inseminating semen into uterine lumen (B).

3.3.3 Pregnancy diagnosis

Pregnancy was detected by a real time B-mode ultrasonography (transcutaneous probe: HS-2000, Honda Electronics Co., Ltd., Japan) at 60 days after insemination.

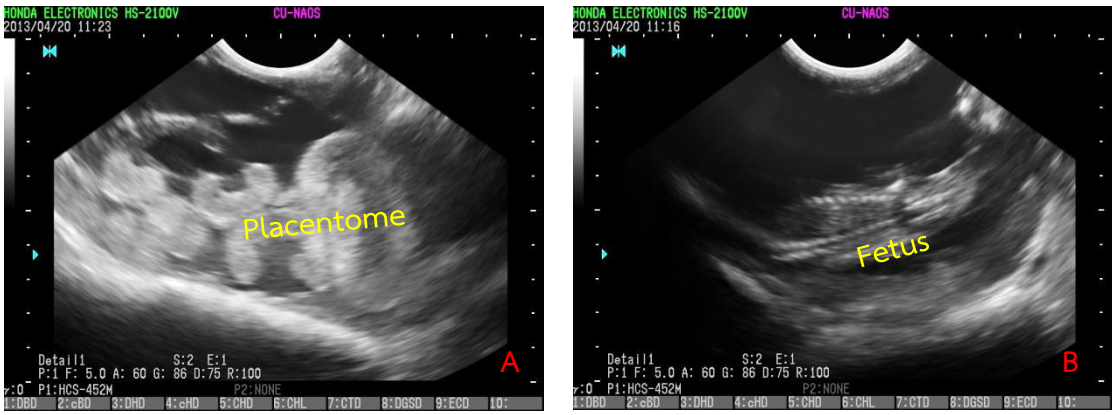


**Figure 9** Ultrasound images at 8 weeks of gestation. Placentomes (A) and fetus (B) are seen into uterine lumen of pregnant ewe.

3.3.4 Experimental design

Experiment 1 was designed to evaluate semen quality after freezing and thawing in different semen extenders supplemented with different types of sugar. The experiment was designed into 2 consecutive experiments and was repeated for 9 replicates. Details of the 2 consecutive experiments are as follows:

Experiment 1.1: To compare the post-thawed ram sperm quality among four types of single sugars. The testing extenders used in this part contained 30 mM of glucose (Glu), fructose (Fru), sucrose (Su), or trehalose (Tre).

Experiment 1.2: To examine the effects of combined sugar on the freezing ability of ram spermatozoa. The testing extenders used in this part contained 30 mM of mixed sugars by combining 15 mM of fructose, sucrose or trehalose. The extender containing 30 mM of the sugar selected from experiment 1.1 was used as a control.

Experiment 2 was focused on fertility after laparoscopic artificial insemination using the frozen semen with selected extender according to its performance from the Experiment 1.2. The frozen semen was tested compared to fresh semen in hormonal synchronized ewes. Pregnancy rate was recorded

3.3.5 Statistical analysis

All statistical analyses were carried out using Statistical Analysis System (SAS) package version 9.2 (SAS 9.2, SAS Institute Inc., Cary, NC, USA). In experiment 1, the

statistical model was used to evaluate 1 fixed effect (sugar supplementation) while the replicate was set as a random effect. The data of viability, acrosome integrity, and results of motility determined by CASA in each group were expressed as mean ± standard error (SEM). The data were tested for normality by the Shapiro-Wilk test and for homogeneity of variance using Levene test. The quantitative data of treatments were analyzed by one-way ANOVA followed by the Tukey-Kramer test.

In experiment 2, the percentage of fertility rates was analyzed in each treatment with a categorical model (CATMOD procedure). Chi-square test ($\chi^2$) was employed to compare the fertility rates between groups. Differences between experimental groups were considered to be statistically significant when $P < 0.05$.

## 3.4 Results

3.4.1 Experiment 1 Effect of different sugar supplementation on post-thawed ram sperm quality

*3.4.1.1 Effect of sugar types*

The effects of sugars supplementation on the semen freezing extender quality analyzed are presented in Table 2. The semen freezing extenders with fructose or sucrose treatments showed a significantly higher percentage of post-thawed sperm motility when compared with the glucose or trehalose supplementation groups (64.3% or 63.6% v 54.7% or 55.9%, respectively; $P$ <0.05).

**Table 2** Mean percentage of total motility, viability and acrosome integrity for each sugar supplementation in post-thawed ram sperm.

| Extender | Total motility(%) | Viability(%) | Acrosome integrity(%) |
|---|---|---|---|
| **Fructose** | 64.3 ± 7.9[a] | 79.4 ± 11.0[a] | 79.6 ± 6.0[a] |
| **Glucose** | 54.6 ± 5.9[b] | 72.3 ± 12.3[a] | 74.3 ± 7.3[a] |
| **Sucrose** | 63.6 ± 8.7[a] | 76.1 ± 9.0[a] | 78.3 ± 6.4[a] |
| **Trehalose** | 55.9 ± 9.2[b] | 71.8 ± 10.4[a] | 75.1 ± 6.2[a] |

Different letters within same column represent a significant difference (Tukey – Kramer test, $P < 0.05$)

*3.4.1.2 Effect of combined sugars*

The basic semen freezing extender using sucrose and trehalose (ST) analyzed by CASA showed a significant higher motility than the other groups. Mean percentage of viable spermatozoa (Via) at 1, 3, and 6 h of the ST group was greater than the fructose combined with sucrose (FS) and fructose combined with trehalose (FT) groups ($P < 0.05$). At 9 h of incubation time, the ST group had higher ($P < 0.05$) live and intact acrosome spermatozoa than the other groups.

Average path velocity (VAP) at 6 h of storage was significantly reduced ($P < 0.05$) in the Fru treatment compared to all other treatments. Within the ST group, there tended to be a higher percentage of straight-line velocity (VSL) at all times; however, significant difference ($P < 0.05$) was observed at 6 h of storage. The mean percentage of curvilinear velocity (VCL), straightness (STR) and linearity (LIN) did not differ ($P > 0.05$) between the groups during storage, but mean percent of STR in the Fru group at 9 h of storage was significantly lesser ($P < 0.05$) than the ST group (Table 3).

**Table 3** Effect of the sugar combination supplemented into freezing extender on sperm quality parameters analyzed by CASA in post-thawed semen stored for various time periods at 37°C.

| Parameters | Groups | Storage time (h) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1 | 3 | 6 | 9 |
| **Mo (%)** | Fru | 64.3 ± 7.9$^{a}$ | 54.2 ± 6.9$^{a}$ | 45.4 ± 10.6$^{ab}$ | 38.0 ± 9.7$^{a}$ | 24.4 ± 11.0$^{a}$ |
| | FS | 66.8 ± 7.2$^{a}$ | 58.0 ± 8.2$^{a}$ | 50.0 ± 5.7$^{a}$ | 39.9 ± 9.0$^{a}$ | 27.3 ± 9.2$^{a}$ |
| | FT | 61.1 ± 9.4$^{a}$ | 52.4 ± 9.1$^{a}$ | 41.1 ± 15.2$^{b}$ | 34.8 ± 9.6$^{a}$ | 27.9 ± 8.3$^{a}$ |
| | ST | 78.6 ± 5.0$^{b}$ | 70.3 ± 6.5$^{b}$ | 62.3 ± 8.0$^{c}$ | 56.8 ± 7.5$^{b}$ | 43.8 ± 6.9$^{b}$ |
| **Via (%)** | Fru | 79.4 ± 11.0$^{ab}$ | 73.8 ± 10.2$^{ac}$ | 66.9 ± 10.3$^{ac}$ | 57.3 ± 9.7$^{ab}$ | 47.4 ± 9.8$^{a}$ |
| | FS | 79.2 ± 8.5$^{a}$ | 69.9 ± 6.9$^{ab}$ | 60.4 ± 7.3$^{ab}$ | 56.6 ± 8.3$^{a}$ | 47.2 ± 8.1$^{a}$ |
| | FT | 73.8 ± 10.7$^{ab}$ | 64.7 ± 11.1$^{b}$ | 58.2 ± 6.5$^{b}$ | 50.4 ± 5.6$^{a}$ | 41.4 ± 9.7$^{a}$ |
| | ST | 84.4 ± 5.5$^{b}$ | 78.6 ± 7.0$^{c}$ | 73.3 ± 7.1$^{c}$ | 64.7 ± 6.2$^{b}$ | 56.3 ± 7.6$^{b}$ |
| **Acro (%)** | Fru | 79.6 ± 6.0$^{a}$ | 76.4 ± 5.7$^{a}$ | 75.0 ± 5.7$^{ab}$ | 69.6 ± 7.4$^{ab}$ | 65.7 ± 5.9$^{a}$ |
| | FS | 79.0 ± 6.0$^{a}$ | 75.9 ± 6.6$^{a}$ | 72.6 ± 6.4$^{ab}$ | 69.1 ± 6.6$^{ab}$ | 65.4 ± 6.2$^{a}$ |
| | FT | 77.9 ± 5.8$^{a}$ | 73.9 ± 7.8$^{a}$ | 69.6 ± 9.7$^{a}$ | 67.3 ± 8.6$^{a}$ | 63.6 ± 7.9$^{a}$ |
| | ST | 82.3 ± 5.5$^{a}$ | 79.7 ± 5.8$^{a}$ | 77.4 ± 5.0$^{b}$ | 75.1 ± 5.0$^{b}$ | 73.0 ± 5.2$^{b}$ |
| **VAP** | Fru | 93.5 ± 20.6$^{a}$ | 79.7 ± 15.2$^{a}$ | 77.5 ± 14.4$^{a}$ | 62.8 ± 17.9$^{a}$ | 60.8 ± 27.5$^{a}$ |
| **(μm/s)** | FS | 91.7 ± 17.0$^{a}$ | 90.8 ± 19.5$^{a}$ | 89.4 ± 32.1$^{a}$ | 65.0 ± 16.3$^{ab}$ | 47.7 ± 20.1$^{a}$ |
| | FT | 91.5 ± 19.5$^{a}$ | 83.3 ± 15.8$^{a}$ | 74.0 ± 20.4$^{a}$ | 63.1 ± 16.9$^{ab}$ | 49.8 ± 21.4$^{a}$ |
| | ST | 96.8 ± 21.4$^{a}$ | 85.6 ± 18.5$^{a}$ | 83.2 ± 12.1$^{a}$ | 81.7 ± 12.7$^{b}$ | 61.1 ± 23.1$^{a}$ |
| **VCL** | Fru | 168.9 ± 41.4$^{a}$ | 159.1 ± 30.6$^{a}$ | 141.0 ± 26.0$^{a}$ | 114.6 ± 35.8$^{a}$ | 103.4 ± 38.8$^{a}$ |
| **(μm/s)** | FS | 166.2 ± 36.6$^{a}$ | 149.7 ± 43.5$^{a}$ | 135.5 ± 33.6$^{a}$ | 108.0 ± 16.2$^{a}$ | 86.7 ± 29.4$^{a}$ |
| | FT | 140.0 ± 20.6$^{a}$ | 135.9 ± 35.2$^{a}$ | 124.6 ± 34.3$^{a}$ | 112.8 ± 34.9$^{a}$ | 87.1 ± 36.2$^{a}$ |
| | ST | 158.8 ± 50.7$^{a}$ | 152.7 ± 44.6$^{a}$ | 128.4 ± 35.5$^{a}$ | 112.0 ± 16.0$^{a}$ | 92.8 ± 31.3$^{a}$ |
| **VSL** | Fru | 63.8 ± 14.0$^{a}$ | 51.1 ± 9.3$^{a}$ | 49.5 ± 10.5$^{a}$ | 41.8 ± 11.1$^{a}$ | 33.4 ± 9.6$^{ab}$ |
| **(μm/s)** | FS | 61.9 ± 10.6$^{a}$ | 56.3 ± 10.0$^{ab}$ | 55.9 ± 23.9$^{a}$ | 43.7 ± 9.8$^{a}$ | 28.0 ± 9.0$^{a}$ |
| | FT | 60.9 ± 17.4$^{a}$ | 54.2 ± 15.3$^{ab}$ | 46.7 ± 9.2$^{a}$ | 39.9 ± 8.9$^{a}$ | 29.0 ± 10.4$^{a}$ |
| | ST | 71.6 ± 20.1$^{a}$ | 65.0 ± 19.4$^{b}$ | 59.5 ± 13.8$^{a}$ | 56.7 ± 14.0$^{b}$ | 45.1 ± 13.7$^{b}$ |

| Parameters | Groups | Storage time (h) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1 | 3 | 6 | 9 |
| **LIN (%)** | Fru | 37.1 ± 5.6[a] | 35.8 ± 4.2[a] | 35.8 ± 4.9[a] | 35.2 ± 9.4[a] | 34.0 ± 4.8[a] |
| | FS | 36.9 ± 6.7[a] | 37.0 ± 7.5[a] | 38.0 ± 8.1[a] | 38.2 ± 10.7[a] | 37.4 ± 7.8[a] |
| | FT | 39.0 ± 8.4[a] | 41.7 ± 12.7[a] | 33.1 ± 5.3[a] | 38.7 ± 8.1[a] | 39.4 ± 10.8[a] |
| | ST | 33.8 ± 6.6[a] | 40.2 ± 6.4[a] | 37.4 ± 10.2[a] | 40.8 ± 10.3[a] | 40.2 ± 10.8[a] |
| **STR (%)** | Fru | 62.6 ± 5.6[a] | 63.4 ± 13.2[a] | 62.9 ± 10.4[a] | 64.1 ± 7.0[a] | 55.1 ± 13.7[a] |
| | FS | 66.3 ± 12.5[a] | 67.0 ± 7.1[a] | 64.1 ± 7.6[a] | 70.0 ± 7.3[a] | 63.4 ± 9.4[ab] |
| | FT | 59.0 ± 11.5[a] | 66.4 ± 8.9[a] | 61.2 ± 4.8[a] | 64.7 ± 9.3[a] | 63.8 ± 14.7[ab] |
| | ST | 65.2 ± 11.3[a] | 70.2 ± 11.7[a] | 65.4 ± 11.0[a] | 71.0 ± 8.8[a] | 66.2 ± 8.8[b] |

Fru, Fructose; FS, Fructose + Sucrose; FT, Fructose + Trehalose; ST, Sucrose + Trehalose

Different letters indicate significant differences between the data at the same column and parameter (Tukey – Kramer test, $P < 0.05$)

3.4.2 Experiment 2 Effect of sugar combination supplementation on fertility rate after LAI with fresh or frozen semen

After trial of *in vitro* characteristics of post-thawed semen, the TC based extender in the ST group showed greater results than the other treatments. This formula was; therefore, used to compare fertility after LAI with fresh semen.

Of the 70 ewes, three ewes were excluded because they did not show estrus sign after estrous synchronization. Therefore, the results in this study were based on 67 ewes (Table 4). The average time interval between CIDR-G® withdrawal to estrus was 35.3 ± 10.5 h (range 23.4 to 60.0 h). The mean of insemination time after withdrawal of CIDR-G® did not differ between the fresh and frozen groups (57.0 versus 58.6 h; $P > 0.05$). At the timing of insemination, the average number of dominant follicles (diameter > 7 mm.) per ewe was 1.7 follicles.

Fifty-six of 67 ewes (83.6%) were confirmed pregnant after insemination using ultrasonography. There was no significant difference in pregnancy rate between the frozen and fresh semen (82.9 v 84.4%) when using the LAI technique (Table 4).

**Table 4** Effect of LAI with fresh or frozen ram semen on pregnancy rates.

| Group | No. of ewes inseminated | No. of ewes pregnant at Day 60 (%) |
|---|---|---|
| Fresh semen | 32 | 27/32 (84.4%) |
| Frozen semen | 35 | 29/35 (82.9%) |


**Figure 10** Lambs born by laparoscopic artificial insemination with Corriedale/ Bond (A) or Dorper (B) rams semen.

### 3.5 Discussion

As its chief function, exogenous sugar in a semen freezing extender served as an energy source for spermatozoa. In the present study, four different types of sugars were compared in the efficiency of ram semen cryopreservation. The authors found that these sugars affect post-thawed semen qualities differently. The post-thawed ram semen qualities were increased in the fructose group than the other sugars. This result agrees with previous reports that fructose is the best of the supported extenders for semen cryopreservation in dogs and rams (Salamon and Maxwell, 2000; Ponglowhapan et al., 2004b). Therefore, it is suggested that the fructose concentration used in this study may act as sperm support (Fiser et al., 1987; Salamon and Maxwell, 2000). In small ruminant seminal plasma, fructose is the primary substrate for glycolysis (Glover, 1956; Pellicer-Rubio et al., 1997). The end product of glycolysis pathway produces more ATP, which supports sperm motility. In freezing extender, fructose produces ATP better than glucose, because glucose is converted to fructose before glycolysis (as presented as Figure 11). Therefore, the post-thawed sperm motility in the fructose group in this study was significantly higher than the glucose supplementation.

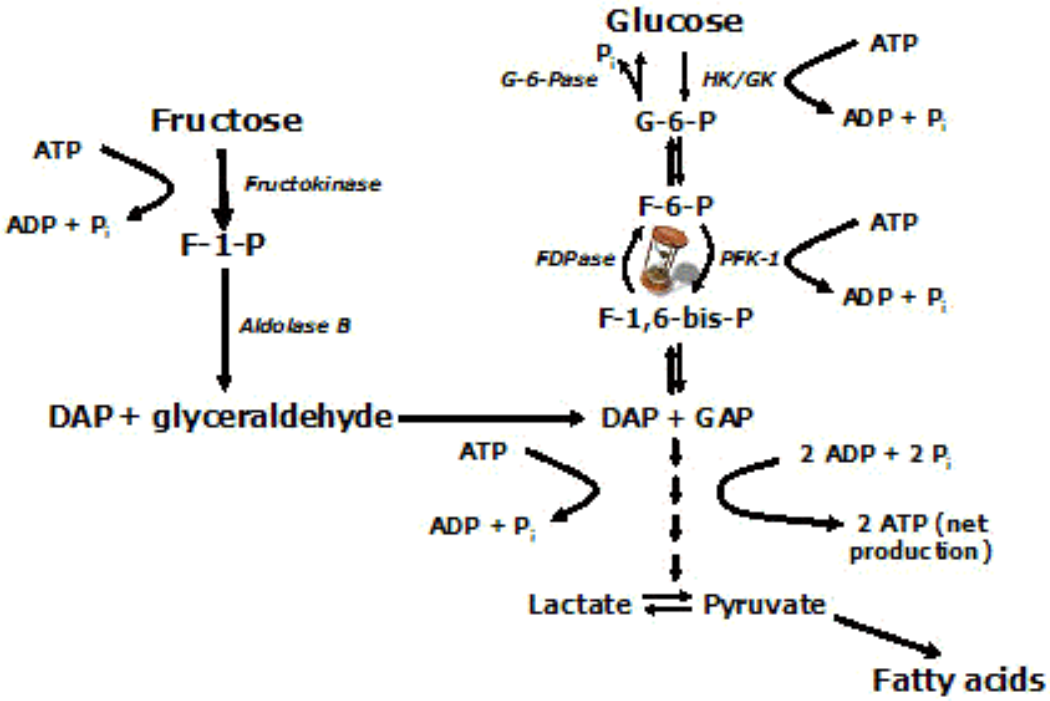


**Figure 11** The pathway of glycolysis of fructose or glucose to pyruvate and energy production of ATP (modified from http://www.medbio.info/horn/time%2012/carbohydrate_metabolism%20march%202007a.htm).

Sugars can help spermatozoa not only as an energy source, but also as a spermatozoa protective action within the cryopreservation process. As temperatures drops, more ice crystals in a spermatozoa cell are formed and caused injury to the plasma membrane in the spermatozoa called 'Cryoinjury'. It induces cell stresses with osmotic pressure changes and leads to death of frozen cells (Watson, 2000; Pegg, 2010). Regarding plasma membrane, it is the outer structure of spermatozoa which acts as a protective barrier of the spermatozoa and can be destroyed by semen cryopreservation, resulting in the detrimental effects on its functions, including sperm fertilizing process (sperm capacitation, acrosome reaction and sperm-oocyte fusion) (Parks and Graham, 1992). Destruction of the plasma membranes affects viability, longevity and fertility of post-thawed spermatozoa. From previous findings, monosaccharide (glucose or fructose) can be permeable to

the membrane because of its small molecular weight. However, many additive agents in semen freezing extenders, including glycerol and Equex STM paste, can also be effective in protecting sperm plasma membrane (Akourki et al., 2004; Anakkul et al., 2013b).

Disaccharides are capable of protection the lipid bilayer of cryopreserved spermatozoa through the hydrogen bonding of molecules and; thus, prevent the growth of ice crystals (Uchida et al., 2007). In addition to above mechanism, trehalose decreases the supply of free water molecules from the solution to spermatozoa and induces dehydration in the cell, reducing intracellular ice formation. Not only trehalose, but also sucrose can reduce ice crystal formation. However, the inhibitory effect of sucrose on ice crystal growth was lower than trehalose (Sussich et al., 2001).

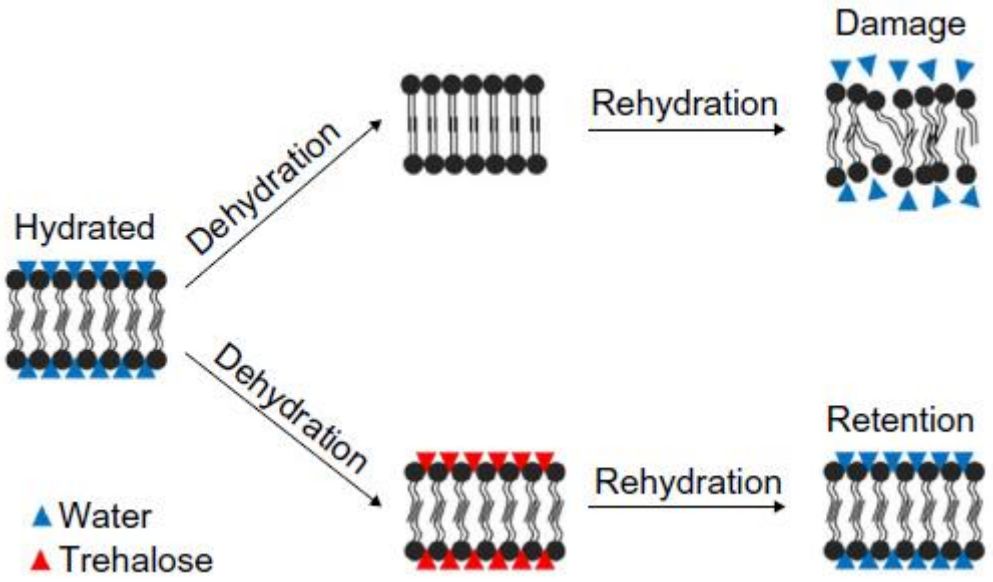


**Figure 12** The diagram represent that how trehalose can effectively stabilize lipid bilayer of cell (Source: Rozsypal (2015))

Sucrose is generally found in plants, while trehalose is principally found in animals. Both of these disaccharides are capable of persistent in low temperatures (Crowe et al., 1988). Both sucrose and trehalose can be found in natural CPA, which contains fructose or glucose rings linked by the glycosidic bond. Acid hydrolysis can

break this bond and convert sucrose into fructose and glucose. In the TC solution used in this experiment, sucrose was hydrolyzed to fructose and glucose monomers by citric acid (Del Pilar Buera et al., 1995). This may explain the results of semen quality that showed no statistically significant difference between sucrose and fructose in this context. Trehalose is composed of two units of glucose, therefore the post-thawed motility in this research is lower than the fructose and sucrose groups, but similar to the glucose group.

Besides, many studies found that trehalose protected spermatozoa from damage by oxygen radicals (Aisen et al., 2005; Hu et al., 2010; Tuncer et al., 2013). The semen freezing extender containing trehalose enhanced the glutathione peroxidase (GSH-Px) and catalase (CAT) activity in post-thawed ram and goat semen (Bucak et al., 2007; Atessahin et al., 2008). These enzymes defend against reactive oxygen species (ROS) and toxic products of metabolism during the freeze-thaw process, thus showing a marked improvement in sperm motility and viability.

In the current study, the effect of sugar combination on the ability to maintain post-thawed sperm viability and longevity was investigated. The combination of sugars supplemented in a ram semen freezing extender showed higher post-thawed semen qualities than the single sugar. Similar results were also reported in goat, human and monkey (Nagai et al., 1982; Aboagla and Terada, 2003; Si et al., 2006; Khalili et al., 2009; Naing et al., 2010). Our findings showed that over 9 h of incubation time, the ST treatment group provided higher proportion motile and viable spermatozoa than the other treatments. Khalili et al. (2009) also suggested that sucrose combined with trehalose was capable of improving the percentage of motility, viability, and membrane integrity in post-thawed goat spermatozoa.

In this study, we further investigated the fertility rate of cryopreserved ram semen using the semen extender supplemented ST. The data showed an acceptable pregnancy rates at 84.4% after LAI of post-thawing ram semen with ST, which was similar to the fresh semen at 82.8%. The pregnancy results obtained in previous studies ranged between 40.5 to 98.2% (Halbert et al., 1990; Findlater et al., 1991; Gimenez-Diaz et al., 2012).

In summary, the data of this study showed that the combination of sugars supplementation in TC freezing extender proved to be effective cryopreservation for ram semen. This study suggests that sucrose plus trehalose (at the final concentration of 30 mM) is the preferable supplementation which can improve semen quality and provide a high successful pregnancy rate in ram. However, the optimized molarity and ratio of sucrose or trehalose are factors that need further investigation. In addition, the understanding of functionality and positional protection during the cryopreservation process of differential sugar molecules may lead us select the appropriate sugar implementation in semen freezing extender with higher pregnancy rate.

# CHAPTER IV

# A SIMPLIFIED SUPEROVULATION PROTOCOL USING SPLIT-SINGLE ADMINISTRATION OF FOLLTROPIN-V IN HYALURONAN: AN APPLICATION TO PURE BREED SHEEP

## 4.1 Abstract

Superovulation is the important step in assisted reproductive technology in livestock. Regarding the superovulation protocol in ewes, FSH is usually administered twice a day for 3 to 5 d to maintain circulatory levels, which is time and labor consuming. This causes difficulty to initiate reproductive biotechnology at an industrial scale. There is much evidence suggesting that a single superovulation injection with a degradable polymer in ruminant may overcome the limit of traditional protocols. The objective of this study was to determine the efficaciousness of split-single FSH injection with hyaluronan for stimulated ovarian response in ewes. Experiment 1 was performed to compare the number of ovulation, recovered embryos, and fertility rate after embryo transfer between split-single FSH dissolved hyaluronan (S group; 150 mg FSH on the first day and 30 mg 48 h later; n = 21) and six decreasing doses of FSH (M group; 50 50, 30, 30, 10, and 10 mg; n = 22) administered to ewes. Ovarian response and recovered ova/embryo production did not differ significantly between groups. However, grade 1 & 2 collected embryos in S group tended to be higher than in the M group. Experiment 2 aimed to test the effectiveness of a simplified split-single FSH administered at a purebred sheep commercial farm. Using this method, the number of good grade recovered embryos (d2) was 4.8 ± 5.0 and 4.0 ± 2.5 per donors in Corriedale and Bond sheep breeds, respectively. These findings suggest that the simplified technique for superovulation procedure in ewes purposed in this study is effective animal welfare and practical at a commercial farm scale.

## 4.2 Introduction

The sheep industry in Thailand is not a major livestock production industry but is gradually becoming more popular**.** In the last decade, purebred sheep, either wool or/and meat types, have been imported increasingly for crossbreeding or to maintain purebred flocks. However, the cost of transportation and adaption are two major obstacles to enhance production. Using reproductive biotechnology such as LAI or/and ET can possibly overcome these problems. Recently, a high success rate of LAI/ET in high genetic goats raised in tropical conditions was reported (Anakkul et al. 2013). A similar application, through simplified single FSH superovulation in sheep was the aim of this study in order to reduce stress in animals for the application in the Thai sheep industry.

A minimal administration of FSH has been proposed to simplify the superovulation procedure. A single superovulation injection with several degradable polymer in ruminant to sustain hormone release has been previously studied by several researchers (Lopez-Sebastian et al., 1993; Yamamoto et al., 1994; Satoh et al., 1996; Sugano and Shinogi, 1999; D'Alessandro et al., 2001; Kimura et al., 2007; Tríbulo et al., 2012). In bovine, administration of FSH dissolved in various substances, for example, polyvinylpyrrolidone (PVP), aluminum hydroxide gel, and hyaluronan (HA), resulted in an increase in ovarian response when compared with FSH multiple injections (Bo et al., 1994; Yamamoto et al., 1995; Kimura et al., 2007; Tríbulo et al., 2012; Chasombat et al., 2013).

Of several biological polymers, HA is abundantly expressed in many tissues of the animal body. Recently, it was also reported that single or split-single FSH injections dissolved with HA increased the number of transferrable embryos in bovine (Tríbulo et al., 2011; Tríbulo et al., 2012). Previous findings have shown that ovarian characteristics and the number of collected embryos were not significantly different between split-single IM injection of Foltropin-V dissolved in HA (HA-FSH) and traditional twice-daily IM injection groups. To apply an ART to the sheep industry in Thailand, it is necessary to begin with a simplified technique of superovulation

program. It appears that the study on the effects of split-single HA-FSH injection on superovulatory response in ewes has yet to be examined. Therefore, the overall objectives of this study were to i) assess the efficacy of the simplified superovulation protocol (a split-single dose of HA-FSH) for embryo production compared to the traditional FSH program (several FSH administrations) and ii) examine the pregnancy rate after embryo transfer to two different superovulation programs. A successful ovulation protocol will further be implied to commercial farms in Thailand to increase the number of offspring born from a low number of pure bred sheep.

## 4.3 Materials and Methods

### 4.3.1 Chemicals

All chemicals in this study were purchased from Sigma-Aldrich Chemical Company (Sigma, St.Louis, MO, USA), unless stated otherwise.

### 4.3.2 Experimental design

Experiment 1 was designed to assess the ovarian responses, hormonal changes, and embryos produced in two different superovulation procedures (split-single HA-FSH v multiple FSH injections) in mixed breed ewes. All animals received either of the following two treatments: Split-single HA-FSH injection (S) or Multiple FSH injection group (M). Next, 22 – 24 h after showing estrus signs, LAI was applied with ram semen in all of donor ewes. Embryos were collected by direct oviductal flushing at 2d after LAI and the transfer to the recipient at the oviduct. Pregnancy was confirmed using transabdominal ultrasonography (HS-2000, Honda Electronics Co., Ltd., Japan) 60d after performing ET (n = 21 and 19 in S and M groups, respectively).

Experiment 2 aimed to validate the efficiency of the application of the simplified superovulation procedure in purebred sheep raise at a commercial scale.

4.3.3 Animals

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Chulalongkorn University (Approval No. 13310050). In experiment 1, forty-three apparently healthy crossbred ewes with a good body condition score (BCS) ranging between 3 – 3.5 (1 – 5 scale) were randomly allocated into two superovulation protocols. Recipients (healthy crossbred ewes; n = 44) were estrus synchronized before receiving embryos from a donor. All animals were housed under natural environmental conditions. In experiment 2, the purebred ewes, including Corriedale and Bond, were kept under ranching conditions at a sheep commercial farm located in western Thailand.

4.3.4 Donor and recipient synchronization protocols (Figure 13)

Regarding the donor group, all animals were superovulated as previously described by Shin et al. (2008) and Mayorga et al. (2011) with minor modifications (summarized in Fig 13). Briefly, estrus synchronization was applied using a CIDR-G device (Interag, Hamilton, New Zealand) for 10 d. In the split-single HA-FSH protocol, a total of 180 mg of FSH (Folltropin-V®, Bioniche Animal Health, Vetrepharm, Ontario, Canada) was prepared by dissolving 10 mg/ml of 750 kDa hyaluronan (MAP-5®, Bioniche Animal Health, Vetrepharm, Ontario, Canada). HA-FSH at doses of 150 and 30 mg were administered in the morning of days 8 and 10 for CIDR-G® implanted ewes.

In the multiple FSH protocol for superovulation, FSH was divided into 6 doses (50, 50, 30, 30, 10, and 10 mg) administered twice daily (12 h interval) in the control group. The first dose of FSH was started on day 8 after insertion of CIDR-G®.

Subsequently, two doses of 125 µg cloprostenol (Estrumate®, Schering-Plough Animal Health, NJ, USA) were intramuscularly administered at the time of the CIDR-G® removal and after 12 h in both animal groups. After that, estrus behavior was detected every 6 h using apronized rams. Two hundred IU of hCG (Chorulon®, Intervet Schering-Plough Animal Health, Netherlands) was given to induce ovulation as soon as the ewes were in estrus.

All recipients were estrus-synchronized using insertion of CIDR-G® for 10 d and an injection of 300 IU eCG (Folligon®, Intervet Schering-Plough Animal Health, The Netherlands) at the time of implant removal. Estrus was detected after progesterone removal every 6 h using apronized rams. Timing of estrus was recorded.

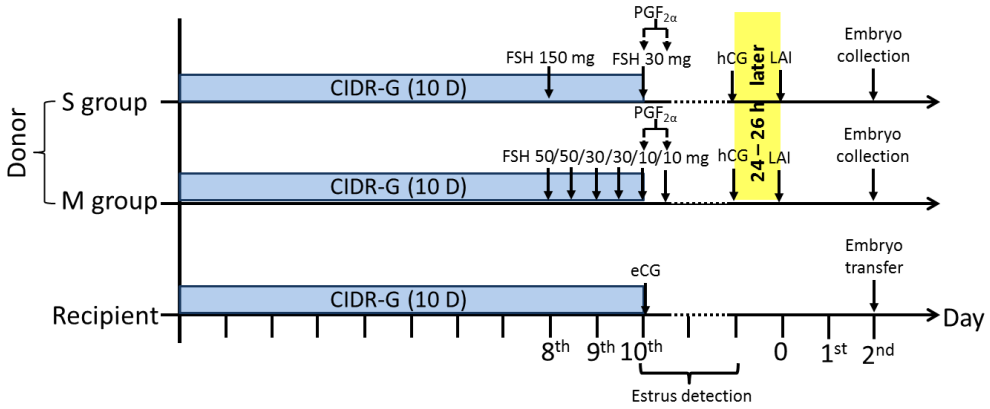


**Figure 13** Diagrammatic schemes of donor superovulation protocols in spilt-single (S) and multiple (M) FSH injection groups and the recipient synchronization program.

4.3.5 Laparoscopic artificial insemination

The donor ewes were inseminated by LAI as described previously 24 – 26 h after showing estrus signs (Panyaboriban et al., 2015). Briefly, they were intravenously anesthetized with ketamine HCl (1.1 mg/kg) combined with xylazine HCl (0.025 mg/kg) and given 0.04 mg/kg phenylbutazone for an analgesic. Total 100 × $10^6$ frozen-thawed spermatozoa were inseminated into the middle of the uterine horn possessing antral follicles under an observation via a direct view 5 mm laparoscope (Schölly, Denzlingen, Germany).

4.3.6 Embryo flushing and evaluation

The embryos were collected by direct oviduct flushing at day 2 following insemination with 10 ml of flushing media (Dubecco' s phosphate-buffered saline (DPBS, Gibco, USA) supplemented with 2% (v/v) fetal bovine serum (FBS, JR Scientific, Inc., Woodland, USA)). The ewes were sedated and anesthetized as described in LAI, and flushing was conducted as previously described by Anakkul et al. (2013a). In

brief, the reproductive tract of the ewe was accessed through a mid-ventral incision. The number of recovered embryos or ova was recorded in relation to the numbers of corpus luteum (CL).

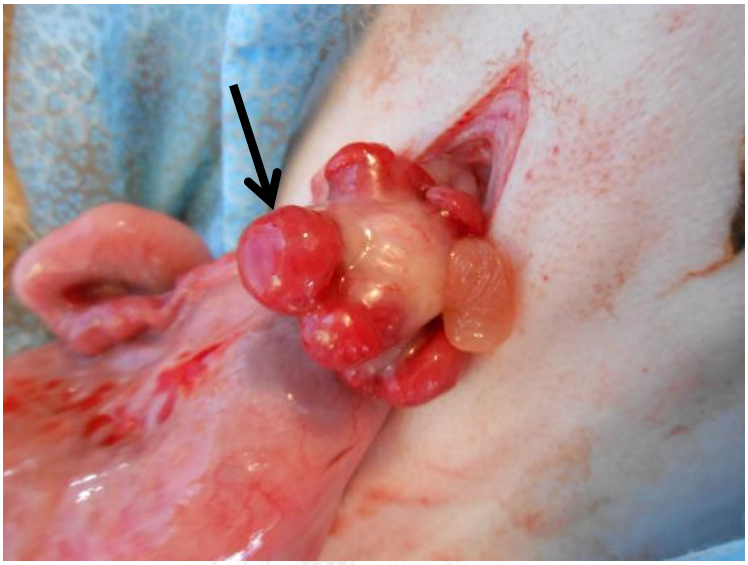

**Figure 14** Superovulary responses on Corriedale donor's ovary. The arrow indicates the CL (Ovulation site).

Regarding oviduct flushing, the flushing medium was infiltrated at about 1 cm above uterotubal junction (UTJ) to the fimbria. The flushing fluid 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 embryos were immediately searched under a stereomicroscope (Nikon SMZ645, Japan) with 10X magnification. The recovered embryos were evaluated and graded according to agreed IETS conventions (Wright, 1998).

4.3.7 Blood sampling, plasma progesterone and estradiol-17β assays

To compare the hormonal profile of the two programs, blood samples were collected from the jugular vein and kept in heparinized tubes from 12 randomly selected ewes per group every 2d after the first injection of FSH in both groups. Plasma was separated by centrifugation at 1500 × *g* for 15 min and stored at -20°C

until analyzed. Plasma progesterone and estradiol-17β were determined using the RIA procedure previously described by Kamonpatana et al. (1976).

*4.3.7.1 Validation of RIA: plasma progesterone*

The reliability of this method was tested in three pools of low, medium, and high standard progesterone added to the blank plasma pools. The coefficients of variation (CV) of the three pools of internal control of the assay were 10.26% (0.1 ng/mL), 9.95% (0.5 ng/mL), and 8.60% (1.0 ng/mL), respectively. The CV of inter assay in pools low, medium, and high standard were 13.1%, 14.8%, and 10.3%, respectively. The sensitivity of the assay was 0.03 ng/ml.

*4.3.7.2 Validation of RIA: plasma estradiol-17β*

The reliability of this method for estradiol-17β determination was tested in three pools of low, medium, and high standard estradiol-17β added to the blank plasma pools. The intra- and inter-assay coefficients of variation were 9.48 and 12.05%, respectively. The sensitivity of the assay was 0.36 – 100 pg/ml. The mean ± SD percentage recovery in plasma was 89.44 ± 3.05. The specificity of estradiol-17β antibody (#8932 180381: Dr. R.I. Cox Csira Prospect, NSW, Australia), defined as the cross-reactivity data obtained in the described RIA system, was as follows: Estradiol-17β 100%; Estradiol-17α 0.48%; Estrone 8.40%; and other steroids less than 1.68%.

4.3.8 Embryo transfer protocol

The embryos were transferred to recipients. Only good quality grade 1 or 2 embryos were transferred to recipients when they were in estrus ± 1 d compared with the donors. The process of embryo transfer followed the embryo collection protocols as embryos were transferred into the oviduct. Two *in vivo* embryos retrieved from M or S superovulation protocols were transferred to a recipient randomly.

4.3.9 Pregnancy detection

60 d after transfer, the ewes were pregnancy diagnosed by real time B-mode ultrasonography via transcutaneous probe (Honda HS-2100V, Japan). In pregnant ewes, they were found as fetal body or placental (placentome maturation into a C-shape or a hollow circle) landmarks within enlarged fluid filled uterine sections.

4.3.10 Statistical analysis

The normal distribution of data was checked using the UNIVARIATE procedure (SAS version 9.0; SAS Institute, Inc., Cary, NC). The quantitative data of treatments (no. of follicles, number of CL, no. of ova and embryos,) were analyzed by general linear model (GLM) or, in the case of unequal variation among treatments, the NPAR-ANOVA with Wilcoxon rank-sum test to test whether treatments varied from each other. The rates of recovery and pregnancy were tested for statistical significance by the Chi-square test ($\chi^2$). Plasma hormone levels (progesterone and estradiol-17$\beta$) were analyzed by two-way ANOVA followed by the Turkey-Kramer test. Differences between experimental groups were considered to be statistically significant when $P < 0.05$.



**4.4 Results**

4.4.1 Experiment 1: Simplified HA-FSH superovulation protocol for in vivo embryo production

All ewe donors in both groups (S and M) showed estrous signs after the completion of the superovulation program (43/43). On average, the onset of estrus after CIDR-G® removal in S group (25.7 ± 3.2 h; range 22.7 to 33.1 h) was earlier ($P < 0.05$) than the M group (28.3 ± 4.2 h; range 22.8 to 35.7 h).

Mean plasma progesterone and estradiol-17$\beta$ hormone profiles according to day of superovulation protocol (day 0: day of first dose of FSH injection) and group are shown in Figure 15. On day 8, the group S ewe donors with split-single injection of HA-FSH had significantly higher plasma progesterone concentrations compared to

the M group (7.4 ± 2.8 ng/ml v 4.5 ± 2.4 ng/ml, $P < 0.001$). In both groups, plasma estradiol-17β levels increased gradually after the first FSH injection. However, there was not significant difference in progesterone levels on day 2, 4, 6, and 10 between the two groups. The averages of plasma estradiol-17β were also not significant between groups. However, the peak of estradiol-17β levels in the S group (2.5 ± 0.8 pg/ml) tended to be higher than in M group (1.2 ± 0.3 pg/ml) at LAI period (day 6).

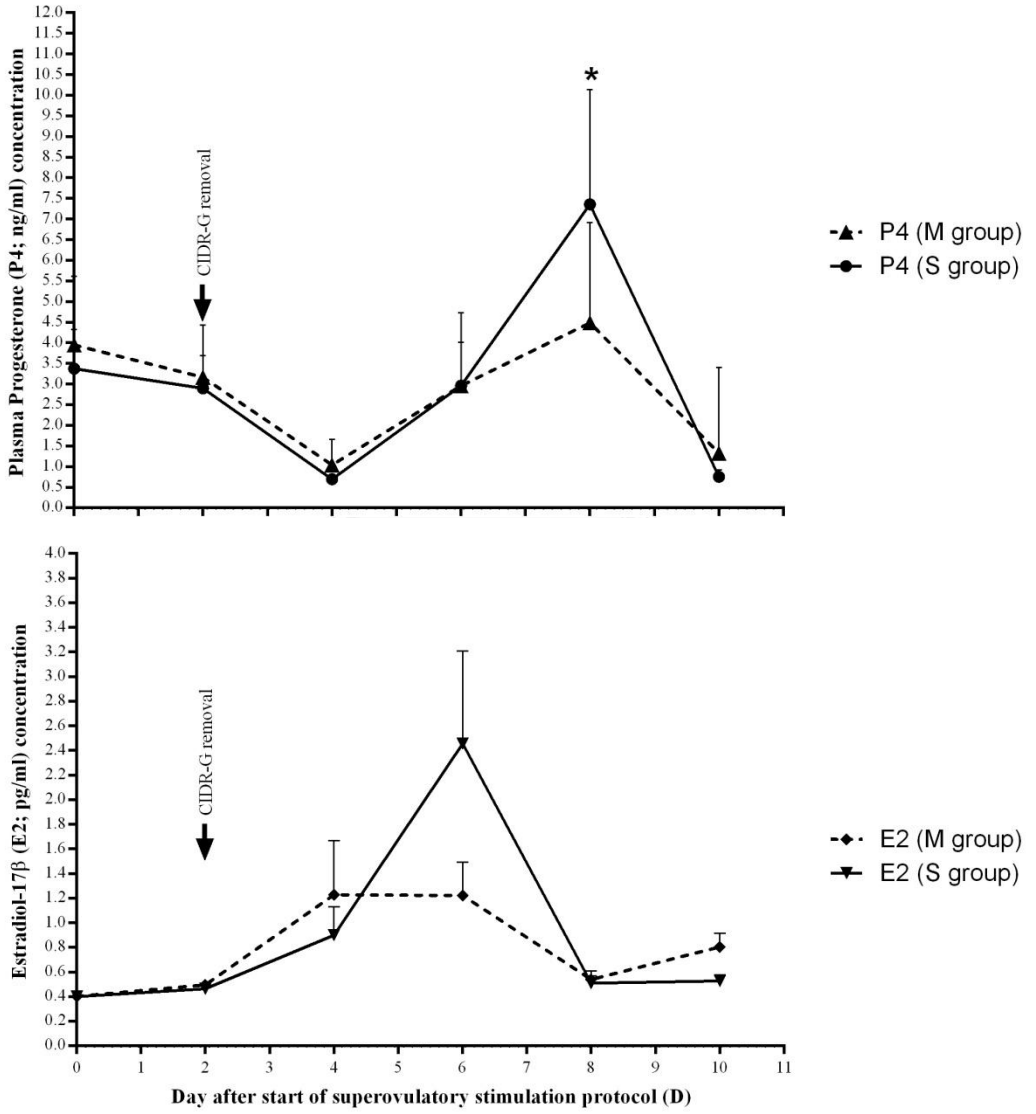


**Figure 15** Peripheral plasma concentrations of progesterone and estradiol-17β of superovulated donor ewes in split-single or multiple injection groups during superovulation, * indicates a significant difference ($P < 0.05$).

For the ovarian responses, there was no difference in an average number of ovulations (i.e., mean number of CL) and average number of recovered ova/ embryos ($P > 0.05$) between two superovulation protocols (Table 5). However, it seems that the S group showed a trend towards an increase of mean number of embryo (5.2 v 3.9 embryos/animal) and fertilization rate (52.0% v 40.9%) when compared to the M group. Mean number of unovulated follicles ranged from 1.7 to 2.1 with no significant difference between treatments (S or M groups; $P = 0.386$). The recovered ova/embryos did not differ between S and M groups (96.1% v 93.1% respectively). In addition, 4 of 22 (18.2%) ewes in the M group did not produce embryos compared with 3 of 21 (14.3%) in the S group.

Regarding the recipient group, 90.9% (40/44) of the ewes exhibited estrus within 48 h following estrus synchronization. Following the embryo transfer from either S or M protocol, the pregnancy rate with embryos from the S group was higher than from the M group (90.5% (19/21) v 78.9% (15/19); $P < 0.05$, respectively).

**Table 5** Numbers of ovulation, collected ova and embryos, and fertilization rates from superovulated ewe donors with split-single or multiple FSH administrations. Data presented in Mean ± SEM.

| Treatment group | S group | M group | *P*-value |
|---|---|---|---|
| No. of ewes | | | |
| treated | 21 | 22 | - |
| with estrous signs | 21 (100%) | 22 (100%) | - |
| positive response | 21 (100%) | 22 (100%) | - |
| ovulations | 10.2 ± 3.4 | 10.8 ± 4.7 | 0.67 |
| ova/embryos per animal | 9.9 ± 3.6 | 10.5 ± 5.2 | 0.66 |
| fertilized embryos per animal | 5.2 ± 5.0 | 3.9 ± 3.5 | 0.32 |
| grade 1 & 2 embryos | 5.1 ± 4.9 | 2.9 ± 2.9 | 0.08 |
| % fertilized per animal | 52.0 ± 42.4 | 40.9 ± 31.5 | 0.33 |

4.4.2 Experiment 2: Application of a simplified superovulation (split-single FSH injection) procedure to commercial farms

To increase the purebred sheep at a commercial farm, the protocol of simplified single FSH treatment combined with embryo transfer was carried out. Two breeds of wool (Corriedale and Bond) ewes were selected for this study. The data were analyzed from 15 Corriedale and 7 Bond sheep, as donors. All animals showed estrous signs after CIDR-G® withdrawal. There was no difference in the timing of estrus between breeds. Thereafter, all the ewes showed positive ovarian responses. The data of ovarian response, embryo production, and pregnancy rate in each breed are shown in Table 6. The number of recovered good grade (1 & 2) embryos per superovulated donor was 4.8 ± 5.0 and 4.0 ± 2.5 in Corridale and Bond, respectively. Following embryo transfer, 15 out of 22 recipients with transferred Corriedale embryos established pregnancy, while 13 out of 14 recipients became pregnant after transfer of Bond embryos. In addition, 28 surplus Corriedale embryos were cryopreserved.

**Table 6** Ovarian responses, embryo production, and pregnancy rate in Corriedale and Bond sheep breeds after superovulatory stimulation with split-single FSH administration protocol.

| **Breeds** | **Corriedale (n = 15)** | **Bond (n = 7)** |
|---|---|---|
| **CL at flushing time** | 9.9 ± 4.9 | 7.8 ± 1.0 |
| **ova/embryos per animal** | 9.0 ± 5.1 | 6.5 ± 1.7 |
| **fertilized embryos per animal** | 5.0 ± 5.2 | 4.0 ± 2.5 |
| **grade 1 & 2 embryos** | 4.8 ± 5.0 | 4.0 ± 2.5 |
| **number of recipients** | 22 | 14 |
| **pregnancy rate (%)** | 15/22 (68.2%) | 13/14 (92.8%) |
| **number of lambs born** | 20 | 20 |
| **number of frozen embryos** | 28 | 0 |

**Figure 16** Lamb born by embryo transfer with in *vivo* embryo production from Corriedale donor.

### 4.5 Discussion

The present study validated the efficacy of a simplified superovulation protocol (a split-single dose of HA-FSH) for embryo production compared to the traditional multiple FSH injection. Moreover, the embryos produced by this procedure are competent to generate pregnancy. These findings help to simplify superovulation protocol when using FSH, which traditionally needed to be administrated every 12 h for 6 – 8 times to effectively stimulate superovulation (Cognie, 1999). Such a traditional procedure can also cause animal stress as well as be time consuming and labor intensive, which make it inappropriate for commercial operations.

Using a single injection of FSH in different diluted degradable agents such as propylene glycol (PG), polyvinylpyrrolidone (PVP), aluminum hydroxide gel, or hyaluronan was previously tested in cattle and ewes (Lopez-Sebastian et al., 1993; Dattena et al., 1994; Yamamoto et al., 1994; Takedomi et al., 1995; D'Alessandro et al., 2001; Kimura et al., 2007; Tríbulo et al., 2012). Although these agents effectively

cause a slow release of circulatory FSH to induce a superovulatory response, evidence has shown that PG and PVP has caused allergic reactions in humans (Catanzaro and Smith, 1991; Gonzalo et al., 1999; Yoshida et al., 2007; Marques et al., 2011) while aluminum hydroxide induced neuropathological reactions and macrophagic myofascitis in humans (Gherardi et al., 2001; Shaw and Petrik, 2009). On the other hand, there have been no reports of side effects from HA administered in mammals. HA, a naturally polysaccharide compound, is found in the extracellular matrix, especially of connective tissues. It has been shown to be well suited for use in medical application such as in tissue engineering (Radice et al., 2000). Similar to the present study, there was no allergic reaction at the injection site and no systematic effects in any of the experimental ewes.

The controlled slow releasing hormone/ drug mechanism of HA was studied (Kim and Park, 2002; Lehr and Haas, 2002; Cho et al., 2003; JungáChung and GwanáPark, 2010; Oh et al., 2010; Upadhyay et al., 2010). Interaction between HA and a deliverable agent was bound by a multivalent anionic charge, diffusing hormones in each layer of the HA polymer surface. At the injection site of the combined HA and agent, body temperature could slowly break the HA structure in each layer and, thus, cause agent release into blood circulation (Cascone et al., 1994; Luo et al., 2000). HA was successfully used for superovulation by single injection with slow FSH release in cattle (Tríbulo et al., 2011). However, in this preliminary study, which attempted to use a single FSH injection for superovulation, the ovarian response was poorer when compared to a traditional FSH multiple injection protocol. The result was similar to previous reports (Alvarez et al., 2010; Tríbulo et al., 2012), which suggested that an additional sub-dose injection of FSH after 48h of single administration in cows had a higher number of ovulation (mean number of CL) than a single injection. Based on this pilot experiment, this study hypothesized that the lack of effect of a single injection with HA likely occurred because of poor FSH release from a single injection of HA-FSH, resulting in less follicular development. Thus, a superovulation protocol in ewes using a split-single administration of HA-FSH was implemented in this study. The overall data showed that split-single IM injection of FSH dissolved in 10 mg/ml

of HA was highly effective in inducing ovarian superstimulation, similar to the traditional multiple FSH injection protocol.

This study was then designed to validate and verify the efficiency of the split-single HA-FSH injection on superovulatory responses and *in vivo* embryo production in ewes. It is noted that the onset of estrus was shorter ($P < 0.05$) in the S group than the M group. This result is similar to a preliminary study in which a split-single injection of HA-FSH in ewes had a shorter interval of progesterone removal to estrus (26.9 ± 3.5 h) compared to other groups (multiple FSH injection: 27.8 ± 6.2 h; single HA-FSH injection: 28.0 ± 4.2 h). This may be an additional advantage of the split-single HA-FSH protocol that could be used as a simplified preparation of donor ewes for fixed-time insemination. However, the mechanism of this phenomenon is still unclear, but it is likely to be at the sex steroid level. The split-single HA-FSH treated ewes had a higher peak of estradiol-17β level than the control group. Commonly, the threshold level of estrogen is required to trigger estrus and LH surge (Evans and Robinson, 1980).

The efficiency of producing good quality recovered embryos was better in the S group compared with the M group. The continual release of exogenous FSH in HA may constantly stimulate the small follicles to develop in the optimized environment. Then, good quality and mature oocytes in these follicles should continue to ovulate and be ready to be fertilized (Thomas et al., 2003). Additionally, it was found that 2 donors (9.1%) in M group had a low ovulation rate (< 5 CL), whereas the range of CL found in the S group was 5 – 16.

Similar to the ovulation rate, the plasma progesterone profile demonstrated that the S group had a significantly higher progesterone concentration than the M group on day 8 (2 d after oviduct flushing). Goto et al. (1988) reported that progesterone level was significantly related to quality and quantity of collected embryos in superovulated cattle. In agreement with Sharma et al.(1993), they showed the positive correlation between superovulatory responses (ovulation rate, recovery rate, and number of embryos) and progesterone level during superovulation in ewes. Furthermore, plasma progesterone profiles for each animal injected with split-single HA-FSH were consistent among its group. This implies that the consistent

efficacy of the synchronizing estrous cycle by split-single HA-FSH, which is useful for set up of an ET operation unit. The result of embryo transfer, then, was related to the treatment, with a higher pregnancy rate found with recipients that received embryos from the S group when compared to those that received them from the M group.

In conclusion, the model of superovulatory synchronization – LAI and ET was validated for use in the sheep industry in Thailand, in particular, with a high genetic merit sheep breed. The simplified superovulation protocol with split-single FSH injection dissolved in 10 mg/ml 750 kDa HA can be safely use for successful superovulatory stimulation of sheep with less animal handling labor and stress. Overall, the results of the present study imply that the split-single FSH injection with HA can be applied to the Thai sheep industry. According to the trial conducted at two commercial farms, it was found that the simplified superovulation protocol can successfully stimulate ovaries to ovulate and produce embryos in Corriedale and Bond breeds. However, further investigation is needed to explore the optimal biotechnology challenge of imported purebred sheep under tropical conditions, particularly in Thailand. In addition, the superovulation procedure developed in the domestic ruminant can also be applied to nondomestic caprinae species and other antelope such as goral, ibex, babary sheep, or deer. However, various steps are warranted to validate independently the protocols for each wild species such as estrus synchronization and estrous detection techniques before insemination.

# CHAPTER V

# GENERAL DISCUSSION AND CONCLUSIONS

## 5.1 Conclusions

In recent years, sheep farming is becoming ever more popular in Thailand. Sheep are abundantly purpose animals, raised for their meat, wool, milk, blood, hides and skins, including promote tourism as well (as summarized in **Chapter 1**). Demand for sheep highly genetic merit breeder has steadily increased for improving farm productivity. Animal breeders have mainly used to develop tools of genetic and productivity improvements in agricultural farms (Marshall, 1994; Kosgey et al., 2006; Kosgey and Okeyo, 2007). In small ruminant farms in Thailand, they need to import highly genetic animal from other countries rather than developing them in their local breeds for genetic improvement. However, the current mean of genetic improvement in sheep can be achieved by crossbreeding through natural mating with a higher genetic male. This is often time-consuming and progress slowly due to a moderate reproductive efficiency.

The suitable breed of sheep for raising in tropical area like Thailand is still not well determined. Since 2010, the Department of Livestock Development, the Ministry of Agriculture and Cooperative and Thai farmers imported Dorper, breed with a highly growing meat-producing sheep from South African. Not only meat-producing breed but also wool-producing (such as Corriedale, Bond, Merino) breed have become popular in recent time in Thailand. However, wool-producing sheep management in Thailand is the major objective for either fine wool production (especially in Maehongson province) or sheep display in resorts.

It is well known that sheep are seasonal breeders whose reproductive activity and functionality are associated with photoperiod and season (Ortavant et al., 1988; Clarke et al., 2009 and described in **Chapter 2**). In rams, several reports have linked seasonality and environmental temperature effects to semen traits (volume,

concentration, motility, and percentage of normal spermatozoa) and male reproductive performance (Godley et al., 1966; Schanbacher and Lunstra, 1976; Ortavant et al., 1988; Rosa and Bryant, 2002; Rosa and Bryant, 2003). A previous research (Anakkul, 2012) revealed that bucks did not show any significant semen characteristics based on various seasons in Thailand. This may different from our case report in Doper in **Chapter 2** that we found azoospermia case in Dorper ram and a poor semen quality found during a high temperature period. The semen quality is related to temperature, THI, and day length in an imported Dorper ram. This result is in agreement with previous reports in Mexico (Méndez Villalobos et al., 2009). The present research found that the semen qualities of imported ram peaked during December to March (low average temperature, THI and day length). Apart from those months, they had poor qualities of ejaculated semen which not acceptable for freezing or insemination. Then, it is noted that using imported male breeder for genetic improvement may encounter the low semen quality from heat stress.

In this thesis, we try to set up the model of genetic improvement through two kinds of reproductive technology; laparoscopic artificial insemination alone or combined with embryo transfer which can accelerate the genetic progress (Figure 17). This technique is widely studied abroad but no report in Thailand, under tropical condition. Moreover, the application under field condition was studied in sheep farm to get more offspring from a number of seed stock like Corredale, Bond, and Dorper.

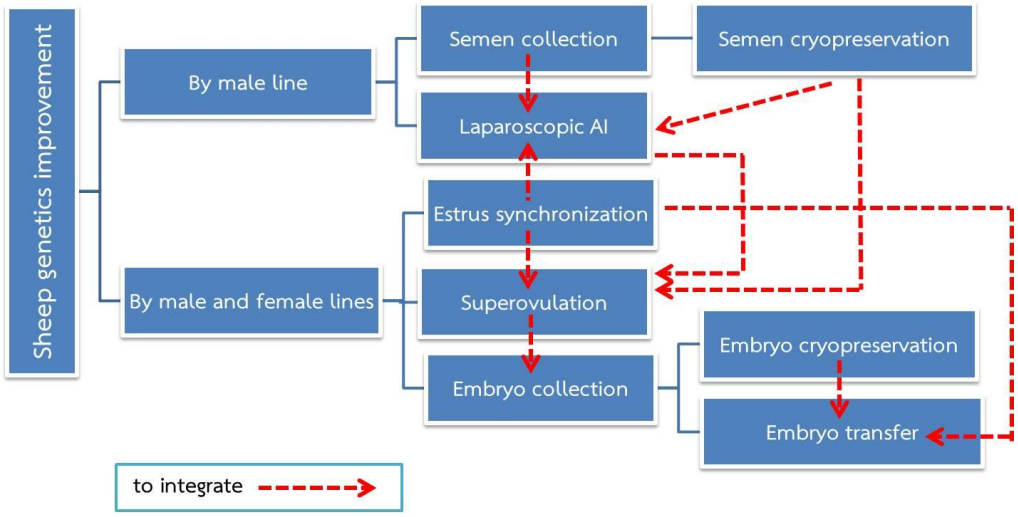


**Figure 17** Model of genetic improvement in sheep industry in Thailand by the ARTs

The first technique to apply in sheep genetic improvement is laparoscopic by fresh or frozen semen. Semen collection should be done in trained ram with artificial vagina. Semen must be evaluated and proceeding to freezing. The frozen semen helped preserving male genetic material and producing offspring when combined with artificial insemination. In general, sheep sperm are sensitive to freezing which both post-thawed semen quality and pregnancy rate after insemination are usually poorer than that obtained from fresh sperm. It has become knowing that cryopreservation causes changes in sperm structure and physiological function such as mitochondria (Windsor, 1996); acrosomal membrane (Pontbriand et al., 1989); plasma membrane (Pérez-Pé et al., 2001); and DNA (Peris et al., 2004). The supplementation of some components especially sugar in semen freezing extender has several beneficial effects on post-thaw spermatozoa. It provides energy support for sperm cell; balances the osmotic pressure of the extender like a cryoprotectant; protects sperm membrane; and defend against oxygen radical damage (Watson, 2000; Ponglowhapan et al., 2004a; Tuncer et al., 2013). **In Chapter 3 (Experiment 1)**, we revealed that supplementation of combination sugars in the freezing extender

improved qualities of post-thawed ram semen than single sugar. Sucrose plus trehalose in the freezing extender gave a better result than other combination and single sugar supplementations by improving not only motility and viability but also acrosome integrity and sperm kinetic parameters (VAP, VSL and STR).

In order to achieve a success of semen cryopreservation, ram semen should be collected in an appropriate time for a proper management of semen cryobanking and insemination (Salamon and Maxwell, 2000). We conducted **Experiment 2 in Chapter 3** to determine the fertility after AI with post-thaw semen collected from Dorper ram as imported from South Africa. We also found that post-thawed semen processed from Tris-citric extender with the addition of sucrose combined with trehalose had a highly fertility rate as same as fresh semen (82.9 v 84.4%, respectively) when combined laparoscopic AI technique. Due to the fact that the anatomy of the sheep cervix is a long, complicate, convoluted and tortuous due to the presence of 4 – 7 cervical rings then difficult to passage of an inseminating gun (Kershaw et al., 2005) to deposit the semen beyond cervix into uterine body. Consequently, transcervical or cervical AI with frozen semen results in poor fertility rates compared to the direct deposition semen to the uterine tract via a LAI (Ritar and Ball, 1993; Eppleston et al., 1994; Sayre and Lewis, 1997). LAI procedure in sheep has been obtained to apply in nearby species such as goat and deer with an acceptable pregnancy rate (McMillan and Hall, 1994; Asher et al., 2000; Baldassarre and Karatzas, 2004). This successful fertilization rate of LAI is caused by the closer deposition of semen directly into the fertilization side (Reinhold et al., 1990). However, the number of motile ram spermatozoa is one factor to limit successful pregnancy rate in LAI (Rodriguez et al., 1988; Eppleston et al., 1994). We found that the fertility after LAI reached success rate when using 80 million total spermatozoa per animal for insemination in sheep.

The second technique to help the genetic improvement in sheep is embryo transfer. The advantage is to improve genetic in both of female and male lines. A good genetic female called “donor” has been superovulated to increase the number of *in vivo* embryos produced per animal. This can be performed by a

supplementation of exogenous gonadotropin like FSH or eCG. More ovulation and embryos produced by one donor can be implanted in a number of recipients. This helps to maximize the female genetic potential. The superovulation program in sheep was widely studied (Betteridge, 2003; Mapletoft and Hasler, 2005; Rahman et al., 2008b; Menchaca et al., 2009). Variability in superovulation response is due to kinds of gonadotropin hormone, additional hormone (progesterone, luteinizing hormone (LH), prostaglandin, etc.), dose, duration, and superovulatory schedule (Ishwar and Memon, 1996; Folch et al., 2001; Rahman et al., 2008a). eCG is generally used in the first place (Cole and Hart, 1930). Superovulation with eCG is cheap and easy to perform because of its only single administration. Although the animal is less stress from this procedure but it's associated with low ovulation rates, poor quality embryos, and/or low pregnancy success due to a long half-life and prolonged antibodies against of eCG hormone in treated animals (Armstrong, 1993; Martemucci et al., 1995; Goulding et al., 1996). Follicular stimulating hormone (FSH) is a better alternative choice than eCG for superovulation. However, one of main problems regarding the practical use of FSH is its relatively short half-life in blood circulation. It has to be given twice a day for 3 – 5 days to stimulate superovulation (Walsh et al., 1993) which is stressful to animals, time consuming and labor intensive and not appropriate when applied in commercial. Several researchers have been developing superovulatory response programs with minimal injection of FSH (Dattena et al., 1994; Yamamoto et al., 1994; Takedomi et al., 1995; Tríbulo et al., 2012; Carvalho et al., 2014).

Our research in **Chapter 4 (Experiment 1)** showed that the split-single FSH administration effectively stimulated in ewes when dissolved in 10 mg/ml hyaluronan (HA; size 750 kDa). In this study, we found whether the half-life of FSH dissolved in HA was prolonged in the blood circulation of treated donor ewes and thus stimulating its gonadotrophic functions in inducing superovulation. Although differences in number of ovulation (CL) and ova/embryos recovered from ewes injected with multiple FSH (6 dosages of FSH every 12h; 50, 50, 30, 30, 10 and 10 mg of FSH) and split-single FSH (150 and 30 mg of FSH) administrations were not

significant, the split-single FSH dissolved HA group tended to result in higher fertilization rate and better embryo quality compared with multiple FSH group. It is achieved with main goal of superovulatory treatments in sheep which is known to produce a high number of transferable embryos and acceptable rate of pregnancy after embryo transfer. Despite other previous studies that have shown the effects of minimal administrations of FSH dissolved other diluents on ovarian responses in ewes such as polyvinylpyrrolidone (Dattena et al., 1994; D'Alessandro et al., 2001) and propylene glycol (Lopez-Sebastian et al., 1993). Nevertheless, these agents have caused adverse effect on animal body, especially injectable site (Catanzaro and Smith, 1991; Gonzalo et al., 1999; Yoshida et al., 2007; Marques et al., 2011). In contrast, HA is a naturally component of the extracellular matrix and can be found in skin, eyes, joints, and almost other tissues. It has been no effect to damage or irritate animal when injected them into animal body. The benefits of this finding is that it can produce in *vivo* embryo from donor ewes with saving time, labor, and reduce animal stress from animal handlings.

Based on our findings **(Experiment 1; Chapter 4)** and the achievements of semen cryopreservation, synchronization and LAI from **Chapter 3**, we established all of these procedures to implement in field condition in the sheep commercial farm in Thailand (as shown in **Chapter 4 experiment 2**). In Thailand, it also directed the strategy of Department of Livestock Development (DLD) of Thailand for increasing population of highly productive sheep breeds. Despite this plan, the development of sheep breed is delayed due to the poor genetic make-up of the local breeds in Thailand. Importation of high merit sheep is a common practice to improve genetic in farms. However, lived animal importing from other country is expensive and more complicated process cause limitation on thoroughly distribution of high genetic merit sheep to small or medium sizes of farms. The ARTs are emphatic reviewed on genetic development programs in sheep industry. It has been the most successful and effective biotechnology and largely responsible for substantial rates of genetic distribution and improvement. Practically, our study demonstrated that these techniques (semen freezing, LAI, split-single FSH superovulation, and embryo transfer)

were successfully performed to apply for produce offspring in sheep farms. It has become an essential part of developed breeding program in sheep. These technologies provided valuable practical opportunities to improve production efficiency and to enhance the valuable genetics in sheep industry in a manner of frog leap.

**5.2 Limitations of the present study and further direction**

One of the objectives of reproductive technology is to improve the genetic improvement. Among them, only two techniques (artificial insemination and/or embryo transfer) are widely used. For AI, it is commonly used in pig and dairy cattle. While the embryo transfer is still limited used in some dairy farms because of its high cost of technique and a lack of expert ET team. Meanwhile in small ruminants, sheep and goats, AI and ET are not studied and not well implemented in Thailand. This dues to a small population in the country and breeding strategy is not well programmed.

This thesis opened the new window of the possibility to implement of LAI and ET in sheep. The previous studies by our team showed the possibility to produce offspring in goats by LAI and ET. Then, this model was applied with a minor modification in sheep presented in this thesis. Although this study was carefully material, method designed, and conducted, there were some unavoidable weaknesses.

First, the scope of this research is focused on the application of laparoscopic AI and ET to sheep production in Thai farm industry. Also, the appreciative knowledge of this technology in Thai farmers is still limited. Then, to educate the Thai farmers the advantages of these two techniques are the key to success for implementation in commercial scale. Now, the farmer's interests of using ARTs for farm production are gradually increasing. In addition, the successful results after utilize those technologies in the farms had been varied depending on many factors such as farm management, environment, animal heath, individually animal physiological responses, breeds, batch of injectable hormone etc., especially in

embryo production by superovulation procedure. Also, the investment of this technology is still expensive from a cost of hormones that imported from abroad. We cannot guaranty the extra income when farmers use the ARTs in their farms. Therefore, the participation of ARTs in small ruminant industry is not easily increased, especially in small scale of farm size.

Second, the poor production was observed by our study in **Chapter 2**, that the imported Dorper ram was affected by heat stress due to a high THI index. As our experience in working with the Thai farmers, the poor quality semen production was observed in other imported male. More studies in the adjustment of environmental housing (temperature, humidity, and light) may improve the semen quality in imported male.

Third, the semen production of ram cannot well perform for insemination and/or cryopreservation in all of year. The research for LAI (from **Chapter 3**) which resulted in same fertility rate between LAI with fresh and frozen-thawed semen will be advantageous for this problem solving. Thus, the development of sheep semen cryobanking in Thailand will be improved and increase high genetic value lamb production. In addition, the frozen semen is more simply transportation and lower cost than lived animal; it is a power tool for genetic distribution to all over farm scales.

Fourth, the highly efficacious superovulation program is imperative that research for support ET technology should be given more attention. Our study of simplified superovulation which is more practical should be applied for farm animal. Furthermore, this technology may help wild animal conservation especially endangered species but it is also needed to further investigate the responsiveness result in each species.

Finally, the application of reproductive technologies for genetic improvement (semen cryopreservation, AI, superovulation, and ET) will be a great opportunity to improve production of sheep. More research work should explore, then to develop sheep industries in Thailand as it is the important agricultural economy which helps the agriculture and the local meat consumption in the country.

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# APPENDIX

## LIST OF PUBLICATIONS AND CONFERENCE PROCEEDINGS

- Panyaboriban S, Suwimonteerabutr J, Phutikanit N, Swangchan-Uthai T, Tharasanit T and Techakumphu M 2015. Effect of various combinations of sugar supplementation in the extender on frozen-thawed ram semen quality and fertility. Thai J Vet Med. 45(2): 229-237.
- Panyaboriban S, Singh RP, Songsasen N, Padilla L, Brown J, Reed D, Techakumphu M and Pukazhenthi B 2016. Reproductive seasonality and sperm cryopreservation in the male tufted deer (*Elaphodus cephalophus*). Theriogenology. In press.
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