CHAPTER IV
EXPERIMENT 2
PRESERVATION AND BREED PROPAGATION OF THAI-NATIVE GOAT USING TRANSCERVICAL DEEP ARTIFICIAL INSEMINATION TECHNIQUE

4.1 Experiment 2.1: Comparison efficiency of estrus synchronization protocols on conception rate following transcervical artificial insemination (TCAI) with timed AI in Thai-native goat

4.1.1 Introduction

No reproduction technology has contributed more toward the genetic improvement of farm animals than artificial insemination (AI) and AI is usually preceded by estrus synchronization (Al Yacoub et al., 2011). Timed artificial insemination (TAI) requires hormonal treatments that ensure an adequate control of both follicular development and luteal activity to synchronize ovulation. Menchaca and Rubianes (2007) reported the pregnancy rate obtained with a single TAI was higher when insemination was performed at 54 h than at 48 h after hormone withdrawal. Moreover, time of ovulation at 64 h after CIDR device removal (experiment 1.2) this time has important implications in TAI programs when insemination at 54 h after device removal. Synchronizing estrus in goats is aimed at both estrous cycle control for natural breeding or AI (Martemucci and Alessandro, 2011). Estrus synchronization plays a major role in fixed time breeding (Rahman et al., 2008). The most common protocol for estrus synchronization in sheep/goats is based on progestagen/progesterone treatment in the form of intravaginal implants (sponges/the controlled internal drug release, CIDR) (Abecia et al., 2011). This hormonal manipulation that can be used during the breeding and the seasonal anoestrous period, induces a great negative feedback on luteinizing hormone (LH) secretion and, in some instances, may cause spontaneous luteolysis, while after withdrawal of the pre-ovulatory LH surge, ovulation occurs in an almost controlled manner (Amiridis and Cseh, 2012).

Two types of sponges are currently commercially available, based on either flurogestone acetate (FGA), marketed as chronogest (Intervet, Angers, France), or medroxyprogesterone acetate (MAP) (Rahman et al., 2008). Additionally, gonadotropins such as pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), and follicle-stimulating hormone (FSH) have been also used as a means of inducing ovulation and superovulation in farm animals (Stenbak et al., 2003). Intravaginal sponges containing MAP or FGA and CIDR are generally inserted into the vagina for a period of 10-14 days and combined with an injection of equine chorionic gonadotropin (eCG) (formerly known as PMSG) (Zeleke et al., 2005; Vilariño et al., 2010). However, short periods of progestagen sponge treatment for as short as 6-9 days have been reported to be successful in inducing/synchronizing estrus in goats (Fonseca et al., 2005).

Vilariño et al. (2011) reported that the pregnancy rate were 75.3% in goats with a combined treatment of CIDR devices and eCG. However, Romano (2004) reported the kidding percentage recorded following AI in the CIDR, FGA and MAP treatments was no significant difference among groups. Currently the CIDR and subcutaneous implants are preferable than sponges, but costs of CIDR are relatively greater than use of sponge and may hinder widespread use of the CIDR (Holz, 2005; Souza et al., 2011). Therefore, the objective of the present study was to comparison efficiency of estrus synchronization protocols on conception rate following transcervical artificial insemination (TCAI) in Thai-native goats.

4.1.2 Materials and Methods

1) Animals, welfare and management

This experiment was carried out at the small ruminant unit, Department of Animal Science, Faculty of Agriculture, Khon Kaen University, located at 16° 26' N latitude and 102° 50' E longitude, Thailand. The experiment was approved by the Animal Ethics Committee of Khon Kaen University (Reference No. 0514.1.12.2/67). Forty indigenous goats, 8-10 months of age, the females had a body condition score of 2.5-3.0 and a body weight of 17.3 kg, and were fed a maintenance diet (NRC, 1981) with ad libitum feeding of fresh ruzi grass. Clean water and mineral block were provided ad libitum. Animals were vaccinated against foot and mouth disease (FMD), hemorrhagic septicemia (HS) and brucellosis according to the

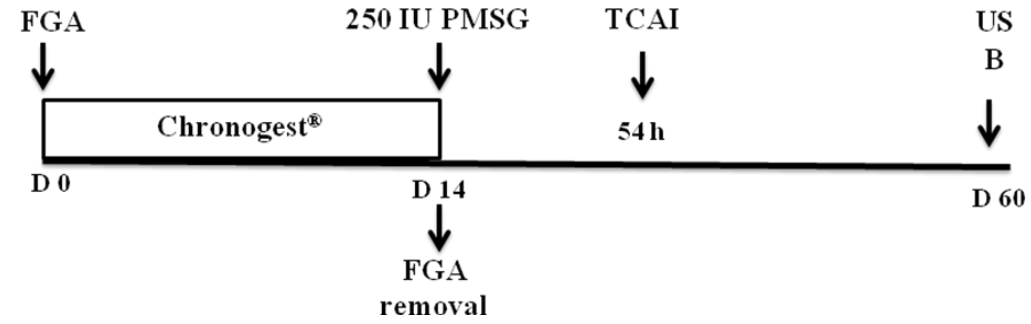
standard farm requirement of the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand.

2) Estrus synchronization and transcervical artificial insemination (TCAI)

All female goats were randomly allotted to one of the two treatment groups. Treatment 1: goats (n=20) were treated with a long-term protocol using an intravaginal sponges containing 40 mg of fluorogestone acetate (Chronogest®; Intervet, Boxmeer, Netherlands) for 14 days and intramusculary injection of 250 IU PMSG (Folligon®, Intervet International B.V., Thailand) at the time of sponge removal. Treatment 2: goats (n=20) were treated with intravaginal CIDR devices containing 0.3 g progesterone (Eazi-Breed™CIDR®, Pfizer, NY, USA) for 14 days. Goats were given (IM) 300 IU injection of hCG (Chorulon®, Intervet International B.V., New Zealand) at the time of CIDR removal. All goats from two treatment groups were transcervically artificial inseminated 54 h after Chronogest® and Eazi-Breed™CIDR® removal with frozen semen from a single proven sire. Each-goat was inseminated with one 0.25 ml mini straw containing 200×10^6 spermatozoa per dose.

TCAI were performed on a breeding rack by lifting the hindquarters of the goat over the top rail while the front legs remained standing on the ground. The speculum was inserted into the vagina and pushed against the tissue surrounding the cervix to help center the external cervical os. AI cather was placed into the folds of tissue surrounding the external cervical os to position the cexvix in the speculum, the catheter was inserted into the cervix. The semen was deposited into cervix more than 5 cm from external cervical os or even into the uterus via the cervix. The catheter was removed slowly, and the speculum was removed. The goat was returned to standing position (Wulster-Radcliffe et al., 2004; Leethongdee, 2009).

Treatment 1: FGA, PMSG and TCAI



Treatment 2: CIDR, hCG and TCAI

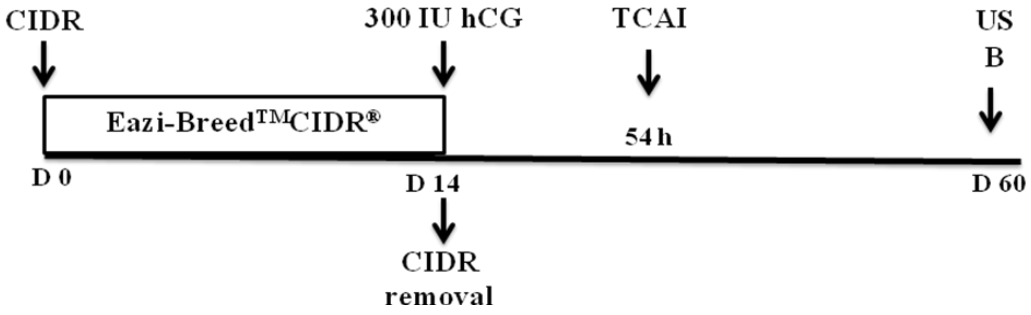


Figure 4.1 Schematic representations comparing the estrus synchronization protocols and transcervical artificial insemination (TCAI) in goat. (CIDR = controlled internal drug release, FGA = Fluorogestone acetate, PMSG = Pregnant Mare Serum Gonadotropin, hCG = Human chorionic gonadotropin, B = Blood sample taken, US = Ultrasonography, D = day, h = hour)

3) Progesterone (P4) concentrations

Blood plasma samples (5 ml) for confirm conception rate were collected via jugular venipuncture on day 60 into an EDTA solutions, then immediately centrifuged at 1500 × g for 15 min. Blood plasma samples were harvested and frozen stored at -20 °C until assayed. P4 concentrations were determined in duplicate by Enzyme-linked Immunosorbant Assay or ELISA (Cushwa

et al., 1992). The mean intra- and inter-assay coefficients of variation were 6.5% and 8.6%, respectively.

4) Conception rate

Conception rate was determined 60 days after insemination by transrectal ultrasonography using a 7.5 MHz transducer (HS-2000, HONDA ELECTRONICS, Japan).

5) Statistical analysis

Percentages of female goats in conception rate were compared between treatments by the Chi-square test. P4 concentrations were compared by a Student *t*-test (Steel et al., 1997). In addition, P4 concentrations were analyzed with a nested analysis of variance with treatment, animal (treatment), and day included in the model (Navanukraw et al., 2004). Data were presented as mean ± SEM, and differences were considered significant when $P<0.05$.

4.1.3 Results

1) Progesterone (P4) concentrations

P4 concentrations on day 60 after TCAI, seven out of twenty goats were determined pregnancy according to high P4 concentrations (>5 ng/ml) for Chronogest® group, whereas six out of twenty were determined pregnancy for Eazi-Breed™CIDR® group (Table 4.1).

Table 4.1 Plasma progesterone concentrations on day 60 after artificial insemination of Thai-native goats

Progesterone concentrations (ng/ml)	n	Chronogest®	Eazi-Breed™CIDR®
1-5	20	13	14
> 5	20	7	6

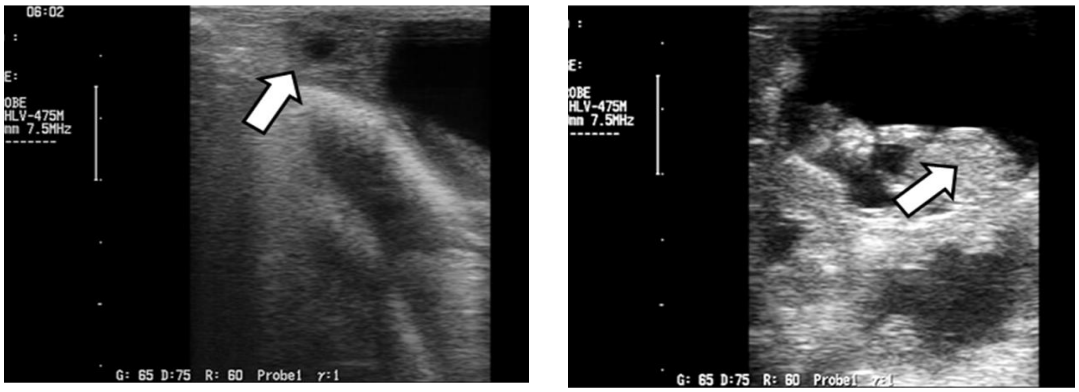
2) Conception rate

The conception rates after TCAI were not significantly different between groups (35% and 30%, for the Chronogest® and Eazi-Breed™CIDR® groups, respectively; P>0.05; Table 4.2).

Table 4.2 Conception rate of Thai-native goats receiving either Chronogest® or Eazi-Breed™CIDR® as determined by ultrasonography

Item	Chronogest®	Eazi-Breed™CIDR®	P-value
No. of goats	20	20	
Conception rate (%)	7/20 (35%)	6/20 (30%)	0.74

The images of non-pregnancy or pregnancy goat recorded on day 60 after inseminated observed by transrectal real time ultrasonography is shown in Figure 4.2.



(a) **(b)**

Figure 4.2 Typical images observed by transrectal real time ultrasonography with 7.5 MHz transducer: (a) uterus of non-pregnant goat, (b) fetal head of goat 60 days pregnant

4.1.4 Discussion

Synchronization protocols, AI-time, seminal state (fresh or frozen semen), and AI techniques significantly affected reproductive outcome (Olivera-Muzante et al., 2011). In the present study was to comparison efficiency of estrus synchronization protocols on conception rate following TCAI in Thai-native goats. The conception rates after TCAI were not significantly different between groups associated with the high P4 concentrations > 5 ng/ml were determined pregnancy (Gaafar et al., 2005). The overall conception rate 60 days following AI and confirmed by P4 concentrations were 35% and 30%, for the Chronogest® and Eazi-Breed™CIDR® groups. This was relatively low when compared to previous findings of Greyling et al. (1994), Motlomelo et al. (2002), Romano (2004) and Greyling and Van Der Nest (2000) in sheep and goats, respectively. This low conception rate could possibly be ascribed to the quality of frozen-thawed semen. Donovan et al. (2004) reported that AI with frozen-thawed semen also gives low fertility. This reduced fertility is partly attributed to damage to spermatozoa during the freeze-thaw process resulting in impaired sperm transport, viability and fertilization capacity (Álvarez et al., 2012; Salamon and Maxwell, 2000).

With transcervical insemination, a low proportion of spermatozoa traverse the cervical canal, meaning a relatively higher number of sperm cells are necessary to get reasonable pregnancy rates (Roca et al., 1997; Baril et al., 1993; Gacitua and Arav, 2005). Laparoscopic intrauterine insemination of goats resulted in improved fertility rates (Ritar et al., 1990; Eppleston et al., 1994); however, the high costs associated with this technique and the necessary qualified inseminators are restrictive factors for the use of laparoscopy to inseminate goats. The TCAI technique is limited due to the anatomy of the ovine cervix (Kershaw et al., 2005). In goats, execution of transcervical insemination is very similar to that in ewes, but much simpler (Cseh et al., 2012). Sohnrey and Holtz (2005) described a method by which semen can be deposited deep into the uterine horns through the transcervical route; the results are at least as good as that achieved by laparoscopy. It was, in all instances, possible to traverse the cervix and a 71% kidding rate was finally recorded.

TAI is an important tool when estrous detection is not feasible, allowing synchronized inseminations and more efficient use of superior males (Menchaca and

Rubianes, 2004) to improve fertility following AI. Motlomelo et al. (2002) who reported that low conception rate could possibly be ascribed to the time of AI (48 and 60 h following progestagen removal) that was late in estrus. However, Menchaca and Rubianes (2007) demonstrated that pregnancy rate for goats with TAI performed at 54 h (63.7%) was higher than for those with TAI performed 48 h (49.4%) after sponge withdrawal.

4.1.5 Conclusion

Both types of synthetic progesterone could be used in Thai-native goat for estrus synchronization and AI. However, price of synthetic progesterone should be considered.

4.2 Experiment 2.2: Comparison efficiency of fresh and frozen semen on conception rate following transcervical artificial insemination in Thai-native goat

4.2.1 Introduction

Artificial insemination (AI) with fresh, chilled or frozen-thawed semen is a basic tool in goat breeding, allowing the diffusion of caprine semen with high genetic value (Leboeuf et al., 2000; Hidalgo et al., 2007). In recent years, artificial insemination with fresh semen has become a common technique in goats (Roca et al., 1997; Leboeuf et al., 2003; Paulenz et al., 2005); however, the commercial use of frozen-thawed semen has been relatively limited in caprine reproduction. As in other domestic animals, the freezing process of semen reduces the viability of the caprine sperm, with a pregnancy rate after AI with frozen-thawed semen, ranging between 7% and 79% (Leboeuf et al., 2003; Nordstoga et al., 2011). However, AI with fresh semen produces conception rates comparable to those obtained with natural breeding (Sohnrey and Holtz, 2005). The biggest obstacle to the exploitation of frozen semen is that the freeze thawing process of goat sperm generally leads to a decrease in the percentage of motile and viable sperm cells after thawing as a result of damage to membrane integrity and ultrastructure (Watson, 2000), particularly associated with low fertility rates after AI (Salamon and Maxwell, 1995; Leboeuf et al., 2000).

The major problem for preservation of buck semen is the components in the seminal plasma, which impair the viability of sperm stored in media containing milk and egg yolk. The toxic interaction with egg yolk is due to an ‘egg yolk

coagulating enzyme' (EYCE), secreted by the bulbourethral gland, which coagulates egg yolk and hydrolyses lecithin to fatty acids and spermicidal lysolecithins (Shipley et al., 2007). Bulbourethral gland secretion also has a toxic interaction with milk. This effect was identified as a 55–60 kDa glycoprotein lipase (BUSgp60), belonging to the pancreatic lipase-related protein 2 family (Sias et al., 2005). This enzyme hydrolyses both triolein and milk triglycerides into free fatty acids that strongly inhibit the motility and damage the membranes of buck spermatozoa. It has been suggested that EYCE and BUSgp60 are the same protein, but this remains under investigation (Parkinson, 2009). As a consequence, diluents for goat semen have either been based upon skimmed milk or, alternatively, the seminal plasma has to be removed before using egg yolk based diluents (Leboeuf et al., 2004). However, washing of the semen is generally time consuming, can affect the sperm viability and promotes sperm loss (Gacitua and Arav, 2005). Therefore, the objective of the present study was to comparison efficiency of fresh and frozen semen on conception rate following transcervical artificial insemination (TCAI) in Thai-native goats.

4.2.2 Materials and Methods

1) Animals and management

This experiment was conducted in the farm of goat farmer, Nong Bua Lamphu Province, Thailand. The experiment was approved by the Animal Ethics Committee of Khon Kaen University (Reference No. 0514.1.12.2/67). Seventy-two indigenous female goats, 8-10 months of age, the females had a body condition score of 2.5-3.0 and a body weight of 17.8 kg, and two mature indigenous bucks (2-3 years old) of proven fertility with average body weight of 30-40 kg. All animals were fed a maintenance diet (NRC, 1981) with ad libitum feeding of fresh ruzi grass. Clean water and mineral block were provided ad libitum.

2) Semen collection and processing

Semen was collected from two mature bucks of proven fertility using an artificial vagina fitted with a graduated test tube. On each collection day, 2-3 consecutive ejaculates (during a time period of approximately 30 min) were collected from each buck. Semen was processed and frozen, as described by (Donovan et al. 2004). Briefly, each ejaculate was assessed for concentration and initial motility. Ejaculates without a minimum concentration 3×10^9 spermatozoa/ml and minimum

initial motility of 3 (scale 0-5) were discarded. Acceptable ejaculates from an individual buck were pooled and then diluted with egg-yolk-glycerol extender (egg yolk 20%, glycerol 7%). After equilibration and adaptation for 2-3 h at 5 °C the semen was rediluted to a sperm concentration of 200 × 10^6 spermatozoa/ml, verified by haemacytometry. The semen was then loaded into 0.25 ml Minitub straws and frozen in liquid nitrogen vapour in a programmable freezer. The temperature was reduced from 5 to -10 °C at rate of -5°C/min, and from -10 °C to -130 °C at a rate of -50°C/min. Straws were then plunged into liquid nitrogen (-196 °C) and stored at this temperature. Random straws from the daily collection of each buck were tested after freezing and if the motility was less than 45-50% then that semen batch was discarded. Prior to insemination/testing, straws were thawed in a water bath at 37 °C for period of 30s (Bispo et al., 2011).

The same two mature male were used to provide fresh semen on the day of insemination. Semen was diluted with diluents of phosphate buffered saline (PBS) to give an insemination dose of 200 × 10^6 spermatozoa (0.25 ml Minitub straws) and held at 5 °C until insemination (within 8 h of collection).

3) Artificial insemination (AI) and conception rate

Inseminations were perfromed using the transcervical artificial insemination (TCAI) technique. All female goats were randomly allotted to one of the two treatment groups. Treatment 1: thirty-six female goats were treated for 14 days with intravaginal CIDR containing 0.3 g progesterone (Eazi-Breed™ CIDR®, Pfizer, NY, USA). Goats were given (IM) 300 IU injection of hCG (Chorulon®, Intervet International B.V., New Zealand) at the time of CIDR removal. Goats were transcervically artificial inseminated 54 h after Eazi-Breed™ CIDR® removal with fresh semen. Each-goat was inseminated with one 0.25 ml mini straw containing 200 × 10^6 spermatozoa per dose. Treatment 2: thirty-six female goats were treated for 14 days with intravaginal CIDR containing 0.3 g progesterone (Eazi-Breed™ CIDR®, Pfizer, NY, USA). Goats were given (IM) 300 IU injection of hCG (Chorulon®, Intervet International B.V., New Zealand) at the time of CIDR removal. Goats were transcervically artificial inseminated 54 h after Eazi-Breed™ CIDR® removal with frozen semen. Each-goat was inseminated with one 0.25 ml mini straw containing 200 × 10^6 spermatozoa per dose.

TCAI were performed on a breeding rack by lifting the hindquarters of the goat over the top rail while the front legs remained standing on the ground. The speculum was inserted into the vagina and pushed against the tissue surrounding the cervix to help center the external cervical os. AI cather was placed into the folds of tissue surrounding the external cervical os to position the cexvix in the speculum, the catheter was inserted into the cervix. The semen was deposited into cervix more than 5 cm from external cervical os or even into the uterus via the cervix. The catheter was removed slowly, and the speculum was removed. The goat was returned to standing position (Wulster-Radcliffe et al., 2004; Leethongdee, 2009).

Conception rate was determined 60 days after insemination by transrectal ultrasonography using a 7.5 MHz transducer (HS-2000, HONDA ELECTRONICS, Japan) and blood samples were taken on day 60 after AI to confirm conception rate. P4 concentrations were determined in duplicate by Enzyme-linked Immunosorbant Assay or ELISA (Cushwa et al., 1992). The mean intra- and inter-assay coefficients of variation were 6.5% and 8.6%, respectively.

4) Statistical analysis

Percentages of female goats in conception rate were compared between treatments by the Chi-square test. P4 concentrations were compared by a Student *t*-test (Steel et al., 1997). In addition, P4 concentrations were analyzed with a nested analysis of variance with treatment, animal (treatment), and day included in the model (Navanukraw et al., 2004). Data were presented as mean ± SEM, and differences were considered significant when $P<0.05$.

4.2.3 Results

1) Progesterone (P4) concentrations

P4 concentrations on day 60 after TCAI, nineteen out of thirty-six goats were determined pregnancy according to high P4 concentrations (>5 ng/ml) for AI with fresh semen, whereas eight out of thirty-six were determined pregnancy for AI with frozen semen (Table 4.3).

Table 4.3 Plasma progesterone concentrations on day 60 after artificial insemination of Thai-native goats

Progesterone concentrations (ng/ml)	n	Fresh semen	Frozen semen
1-5	36	19	28
> 5	36	17	8

2) Conception rate

Data for conception rate for two experimental groups is shown in Table 4.4. The conception rate after TCAI with fresh semen (47.2%) was higher ($P < 0.05$) than that with frozen-thawed semen (22.2%).

Table 4.4 Conception rate for goats inseminated with fresh or frozen semen as determined by ultrasonography

Item	Fresh semen	Frozen semen	P-value
No. of goats	36	36	
Conception rate (%)	17/36 (47.2%)	8/36 (22.2%)	0.03

4.2.4 Discussion

The conception rate obtained following AI with fresh semen was higher than frozen-thawed semen. These results are in agreement with many reports of previous studies that have shown high conception rate when using AI with fresh semen (Karatzas et al., 1997; Donovan et al., 2004; Nogueira et al., 2011). However, the conception rate after AI with fresh semen or chilled semen was low compared to previous reports resulting in an acceptable conception rate values between 60% and 80% (Ritar and Salamon, 1983; Roca et al., 1997; Paulenz et al., 2005). Conceivably, the lower conception rate observed after AI may be attributed to the quality of frozen-thawed semen and the anatomical structure of the cervix (Kaabi et al., 2006). Frozen-thawed semen reduced fertility is partly attributed to damage to spermatozoa during the freeze-thaw process resulting in impaired sperm transport, viability and

fertilization capacity (Álvarez et al., 2012; Salamon and Maxwell, 2000). Cryopreservation is reported to compromise the fertility of goat spermatozoa based on pregnancy rates from AI (Gacitua and Arav, 2005; Purdy, 2006). Roy (1957) stated that semen extenders containing egg yolk in their composition are not recommended for use in goat semen. This being due to the fact that buck seminal plasma contains an enzyme secreted by the bulbo-urethral glands, which in the presence of egg yolk, by hydrolysis, leads to the formation of lysophosphatidylcholines – which are toxic to sperm (Leboeuf et al., 2000). In the search of making the process of cellular conservation more practical and less harmful to sperm, Evans and Maxwell (1987) thus proposed of an extender with a low egg yolk concentration (2.5%) and the low level of egg yolk probably reduced the toxic effect towards the sperm cell during semen cryopreservation (Bispo et al., 2011).

The TCAI technique is limited due to the anatomy of the ovine cervix (Kershaw et al., 2005). Transcervical intrauterine insemination techniques (e.g., transcervical insemination) have been developed and improved to allow the semen to be deposited atraumatically deeply into the uterine horns (Buckrell et al., 1994; Wulster-Radcliffe and Lewis, 2002; Wulster-Radcliffe et al., 2004; Sohnrey and Holtz, 2005). However, the process of manipulating an insemination catheter through the cervix has been linked to reductions in pregnancy and lambing rates. Kershaw et al. (2005) suggests that the alignment of the second or third cervical ring, in relation to the first ring is the principal determinant of depth of penetration and the major impediment to TCAI. It has been suggested that cervical trauma and vaginal/cervical stimulation caused by the catheterization may activate pathways that can interrupt pregnancy at its early stages (Parkinson, 2009). In ewe, the second fold is generally misaligned in relation to the others (Naqvi et al., 2005), and may block the progression of standard AI catheter to within the cervix. This does not happen in other similar species like goat in which the cervical lumen has more folds (Bunch and Ellsworth, 1981; Halbert et al., 1990; Kaabi et al., 2006). Thus, AI in goats can intrauterine insemination via the cervix is much easier than in ewes. Perry et al. (2010) observed that intracervical application of hyaluronan, 52 h after sponge removal improves cervical relaxation, increases cervical penetration and supports transcervical insemination in ewes. The use of an exogenous cervical dilatators in

sheep, such as oxytocin or oestradiol (E2) have been investigated (Khalifa et al., 1992; Stellflug et al., 2001). Khalifa et al. (1992) suggesting that increased secretion of oxytocin and E2 at estrus reduced the difficulty of passing a pipette through the cervix. However, the mechanism of oxytocin-induced cervical dilatation is not known.

4.2.5 Conclusions

The TCAI with fresh semen increase conception rate in Thai-native goat than frozen-thawed semen. However, the conception rate was still low, probably due to the quality of frozen semen and anatomical structure of the cervix. This information warrants further investigation.