Original Article

Glycerol Concentration Effects on Quality and Longevity of Post-thaw Goat Semen

Nitira Anakkul Junpen Suwimonteerabutr Theerawat Tharasanit
Nawapen Phutikanit Jinda Singlor Mongkol Techakumphu*

Abstract

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

Keywords: cryopreservation, glycerol, goat, semen

Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330. Thailand
***Corresponding author: E-mail:** tmongkol@chula.ac.th*

บทคัดย่อ

ความเข้มข้นของกลีเซอรอลมีผลต่อคุณภาพและความยาวนานของการมีชีวิตของอสุจิแพะภายหลังแช่แข็งและทำละลาย

นิธิรา อนัคกุล จันทร์เพ็ญ สุวิมลธีระบุตร ธีรวัฒน์ ธาราศานิต นวเพ็ญ ภูติกนิษฐ์ จินดา สิงห์ลอ มงคล เตชะกำพุ *

การศึกษาครั้งนี้เพื่อพิจารณาผลของกลีเซอรอลที่ระดับความเข้มข้นร้อยละ 7 10 และ 14 ในสารละลายเจือจางน้ำเชื้อสำหรับแช่แข็งต่อคุณภาพน้ำเชื้อแพะแช่แข็งหลังการละลาย ทำการรีดเก็บน้ำเชื้อจากพ่อแพะพันธุ์แบล็ค เบงกอล จำนวน 3 ตัว และทำการประเมินอัตราการเคลื่อนที่ของตัวอสุจิ อัตราส่วนตัวอสุจิมีชีวิต ความผิดปกติของรูปร่างอสุจิ และความผิดปกติของอะโครโซมในน้ำเชื้อสดและภายหลังการละลาย การตรวจประเมินรูปแบบการเคลื่อนที่ของอสุจิโดยการใช้เครื่องตรวจวิเคราะห์อสุจิ ทำภายหลังการละลายและเก็บรักษาที่ 37 องศาเซลเซียส ที่ 0 1 2 และ 3 ชั่วโมง รวมทั้งการผสมเทียมด้วยเทคนิคส่องกล้องลาพาโรสโคปเพื่อทดสอบความสามารถในการปฏิสนธิของน้ำเชื้อภายหลังการแช่แข็ง การศึกษาพบว่ากลุ่มที่แช่แข็งด้วยสารละลายที่มีกลีเซอรอลร้อยละ 10 มีแนวโน้มของอัตราการเคลื่อนที่ของตัวอสุจิ ร้อยละของอสุจิที่มีอะโครโซมปกติ และอัตราส่วนตัวอสุจิมีชีวิต สูงกว่ากลุ่มอื่นๆ โดยเฉพาะที่ 1 ชั่วโมงหลังการละลาย รูปแบบการเคลื่อนที่ของอสุจิมีลักษณะคล้ายคลึงกันในทุกกลุ่มการทดลอง ยกเว้นค่าความถี่ในการส่ายหัวของอสุจิในการเคลื่อนที่ของกลุ่มที่แช่แข็งด้วยสารละลายที่มีกลีเซอ รอลร้อยละ 14 มีค่าต่ำกว่า กลุ่มกลีเซอรอลร้อยละ 10 อย่างมีนัยสำคัญ (p = 0.0211) อัตราการผสมติดจากการผสมเทียมด้วยเทคนิคส่องกล้องลาพาโรสโคปโดยใช้น้ำเชื้อที่แช่แข็งด้วยสารละลายที่มีกลีเซอรอลร้อยละ 10 คิดเป็นร้อยละ 60 (แพะตั้งท้อง 3 ตัว จาก 5 ตัว) ได้ลูกแพะ 3 ตัว สรุปได้ว่าความเข้มข้นของกลีเซอรอลในสารละลายเจือจางน้ำเชื้อสำหรับแช่แข็งส่งผลต่อคุณภาพและความยาวนานของการมีชีวิตของอสุจิแพะภายหลังการทำละลาย สารละลายเจือจางน้ำเชื้อสำหรับแช่แข็งที่มีกลีเซอรอลร้อยละ 10 สามารถป้องกันตัวอสุจิจากความเย็นที่เกิดขึ้นระหว่างกระบวนการแช่แข็งและการละลาย รวมทั้งให้อัตราการผสมติดในเกณฑ์ที่ยอมรับได้

คำสำคัญ: การแช่แข็ง กลีเซอรอล แพะ น้ำเชื้อ

ภาควิชา สูติศาสตร์ เธนุเวชวิทยา และ วิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330
***ผู้รับผิดชอบบทความ E-mail:** tmongkol@chula.ac.th*

Introduction

The application of artificial insemination (AI) is important for genetic improvement programs in goat. However, pregnancy rate following AI with frozen-thawed semen is generally poorer than that obtained from fresh semen (Sohnrey and Holtz, 2005). This indicates that freezing and thawing inevitably induce cryodamage to spermatozoa, thus modification of freezing media to improve the goat sperm viability is required. To date, various freezing media and protocols have been developed aiming specifically at reducing cryoinjury and, in turn, improving the quality of post-thawed spermatozoa. Among factors affecting the freezing ability of spermatozoa, supplementation of freezing medium with various types of cryoprotectant plays a central role in minimizing the physical and chemical stresses occurring during cryopreservation procedure (Purdy, 2006; Peterson et al., 2007). Although it is generally accepted that high concentration of cryoprotectant reduces lethal intracellular ice formation (Mazur and Kleinhans, 2008), this is frequently consequent with probable toxicity of the particular cryoprotectant (Fahy, 2010). Furthermore, other chemical components added into the freezing medium, in particular non-penetrating cryoprotectant such as lactose (Singh et al., 1995; Chang et al., 2006), trehalose (Aboagla and Terada, 2003; Peterson et al., 2007), and surfactants either sodium dodecyl sulfate (SDS) (Aboagla and Terada, 2004[a,b]) or Equex STM paste (Anakkul et al., 2011) have also been reported to affect the freezing ability of goat semen.

Glycerol has three hydroxyl (OH) groups which their hydrogen atoms are likely to form H-bonding with the oxygen atoms of the phosphate groups of membrane phospholipids (Kundu et al., 2000). It acts as a membrane-permeable cryoprotectant, resulting in dehydration of spermatozoa due principally to the osmotically driven efflux of intracellular water (Watson, 1995; Amann, 1999). This prevents the sperm from supercooling and also decreases intracellular ice formation. It is most widely used as a membrane-permeable cryoprotectant for preserving mammalian spermatozoa (Tuli and Holtz, 1994). This cryoprotectant is superior for

protecting buck spermatozoa during cryopreservation to other chemicals (Leboeuf et al., 2000; Purdy, 2006; Peterson et al., 2007). However, suitable amount of glycerol added into the freezing medium remains controversial.

High concentrations (10-14%, v/v) were applied in an early study of buck semen cryopreservation (Ritar et al., 1990) but, at the present time, the glycerol was reduced to 3-9% (v/v) (Leboeuf et al., 2000). Moreover, the amounts of glycerol added into the freezing extender are likely to differ between breeds and individual males (Holt, 2000[b]), for example, in pigs (Waterhouse et al., 2006), marsupials (Taggart et al., 1996) and African antelopes (Loskutoff et al., 1996). The excess amount of glycerol induces osmotic damage to spermatozoa, hence reducing post-thaw semen quality (Holt, 2000[a]). On the other hand, too low glycerol concentration poorly protects the spermatozoa against cryoinjury (Silva et al., 2002). Therefore, appropriate glycerol concentration in the freezing medium, as well as a long-term consequence such as frozen-thawed semen longevity and conception rate following AI should be cautiously determined. In this study, we used semen from Black Bengal bucks as a model since they have high climate-adaptability, fertility and superior skin quality (Husain et al., 1996). However, spermatozoa from Black Bengal buck are quite sensitive to cold stress, and large variability of post-thaw sperm motility (5 to 50%) have been reported (Biswas et al., 2002; Afroz et al., 2008).

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

Materials and Methods

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

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

Semen processing and cryopreservation: Semen from three bucks was collected twice a week using an artificial vagina during May to August 2011 (summer to rainy season). The ejaculate volume was evaluated from a graduated collection tube. Semen pH was evaluated by a pH-indicator paper (Neutralit®, Merck, Darmstadt, Germany). Mass movement and progressive motility of fresh and equilibrated semen were subjectively evaluated under a phase contrast microscope (CX41RF; Olympus, Japan). Sperm concentration was calculated using a hemocytometer (Neubauer, Boeco, Germany) after a 1: 400 dilution with formal saline solution. The ejaculates with an acceptable progressive motility (> 65%) and concentration (> 1,500x10^{6} spermatozoa/ml) were selected and pooled for further processes.

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

Pooled samples were diluted with TCF (1 : 9 at 37^{0}C) and then centrifuged at 940 x g for 10 min at room temperature in order to remove the seminal plasma. The supernatant was discarded, and the sediment containing spermatozoa was divided into three equal aliquots, each was diluted with extender containing 7, 10 or 14% glycerol. The sperm concentration was finally adjusted to 400x10^{6} spermatozoa/ml. The diluted semen was then equilibrated at 4^{0}C for 4 hours. After equilibration, the semen was loaded into a 0.25 ml French mini straw (Minitüb, Landshut, Germany), and the open-end was sealed with polyvinyl powder. The straws were placed horizontally in liquid nitrogen vapor at 4 cm above the liquid nitrogen level in a styrofoam box for 10 min and then plunged directly into liquid nitrogen. Semen thawing was performed at 37^{0}C in a water bath for 30 sec (Deka and Rao, 1987; Leboeuf et al., 2000).

Assessment of semen quality: The fresh, equilibrated and post-thaw samples were assessed for motility, morphology, viability and acrosome integrity. Motion analysis of frozen-thawed semen was additionally performed using the computer-assisted semen analysis (CASA, Hamilton-Thorne Biosciences IVOS, Version 12.3, Bevery, MA, USA). To evaluate the motility of fresh semen, samples were blind-assessed under a phase contrast microscope (100x) by the same technician throughout the experiment. The CASA procedure/setting was performed as the manufacturer's recommendation. The frame rate was set up at 60 Hz (Sundararaman and Edwin, 2008; Bucak et al., 2010). The frozen semen samples to be analyzed with CASA were thawed and diluted (1: 9) with phosphate buffered saline and incubated at 37^{0}C throughout the evaluation process. The motility assessment was carried out at four time points: within 5 min post-thawing (T0), 1 hour (T1), 2 hours (T2) and 3 hours (T3) post-thawing. For CASA, 10 µl

of the diluted sample was placed in a 2X-CEL chamber slide (Hamilton-Thorne, Inc, MA, USA) pre-warmed at 37^{0}C prior to analysis. Three different fields were selected and scanned for motility (MOT, %), progressive motility (PMOT, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/sec), amplitude of lateral head displacement (ALH, μm), beat cross-frequency (BCF, Hertz), straightness (a ratio of VSL/VAP; STR, %) and linearity (a ratio VCL/VAP; LIN, %). The mean value calculated from three analyzed fields was used for statistical analysis.

The viability of the spermatozoa was evaluated following eosin–aniline blue staining (Peterson et al., 2007). The morphology of 500 sperm heads was evaluated after William's staining. The viability and head morphology of spermatozoa were examined under a bright-field microscope (1000x) (Eclipse E200; Nikon, Japan). For tail morphology, a total of 200 spermatozoa fixed in formal saline solution were examined under a phase contrast microscope (400x) (CX41RF; Olympus, Japan). A fluorescein isothiocyanated peanut agglutinin (FITC-PNA) staining was used to evaluate the acrosome integrity as previously described by Axnér et al. (2004), with minor modifications (Anakkul et al., 2011). In brief, 2 μl of sperm suspension was smeared onto a glass slide, and then the sperm membrane was permeabilized with 95% ethanol for 30 seconds. The sperm smear was spread over with the mixture of FITC-PNA (100 μg/ml in PBS) and propidium iodide (PI, final concentration 18 μM) and incubated in a moist chamber at 4^{0}C for 30 min then rinsed with 4^{0}C distilled water before air drying. At least 200 spermatozoa per sample were evaluated using an epifluorescent microscope (BX51; Olympus, Japan). Spermatozoa with intensive bright fluorescent acrosomal cap indicated the acrosome-intact spermatozoa.

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

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

Results

The semen characteristic data of pooled samples were summarized in Table 1. The mean volume per ejaculate of fresh semen from all bucks was 0.59±0.5 ml. Following the addition of freezing extender, the spermatozoa were slowly cooled at 4^{0}C for 4 hours prior to spermatozoa evaluation. It was found that different concentrations of glycerol supplemented in the freezing extender did not affect the spermatozoa quality, in terms of viability, acrosome integrity, percentage of normal morphology (Table 2, $p > 0.05$). There were also no significant differences among glycerol concentrations for post-thaw viability and percentages of normal sperm morphology in all incubation times (Table 2). Nevertheless, spermatozoa cryopreserved with 10% glycerol tended to have a higher percentage of live spermatozoa when compared to other concentrations. This finding coincided with the results that the 10% glycerol preserved integrity of acrosomal membrane better than other concentrations especially when examined at T1. Although MOT and PMOT gradually decreased along the incubation time, the buck spermatozoa frozen with 10% glycerol showed superior results of MOT and PMOT compared to the 7% and 14% glycerol groups in all evaluation times (Table 3). The significant differences were shown at T0 for PMOT and at T0 and T1 for MOT ($p < 0.05$). Motion characteristics in all glycerol groups were similar except that the beat cross frequency of the

Table 1 Semen characteristics of pooled samples obtained from Black Bengal goat bucks

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

spermatozoa frozen with 14% glycerol was significantly lower than the 10% glycerol group (p = 0.0211, Table 3). The spermatozoa frozen with the 10% glycerol protocol retained the fertilizing ability since the ultrasound scanners diagnosed 60% of pregnancy (three of five does) with 100% accuracy. This resulted in a total of three live offspring.

Discussion

Goat sperm are sensitive to cryopreservation as sperm quality and pregnancy are usually poorer than those obtained from non-cryopreserved sperm (Medeiros et al., 2002; Apu et al., 2012). In this study, we found that the amount of glycerol supplemented

Table 2 Mean percentages of viability, acrosome intact, normal head and normal tail morphology of equilibrated and post-thaw spermatozoa frozen with different glycerol concentrations (7, 10 and 14%)

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

Statistical analysis was used to compare the values obtained from different glycerol concentrations. Within the same sperm parameter, different superscripts denote values that differ statistically significant (p < 0.05). SEM indicates standard error of the mean.

Table 3 Motility characteristics of post-thaw spermatozoa frozen in freezing extender supplemented with 7, 10 and 14% glycerol

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

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

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

Moreover, the latter study used subjective sperm evaluation which variations of 30 to 60% have been reported, while this study used CASA, an objective method (Verstegent et al., 2002) which provides more accurate estimates and has demonstrated to be a useful tool to evaluate kinematics properties of individual spermatozoa (Tuli et al., 1992; Mortimer, 2000; Verstegent et al., 2002). On the other hand, poor sperm quality obtained from a high glycerol concentration (14%) probably causes an extreme osmotic stress that can damage the cell structures and impair the sperm motility (Deka and Rao, 1986; Gil et al., 2003; Sönmez and Demirci, 2004; Sundararaman and Edwin, 2008).

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

In conclusion, the glycerol concentration in semen freezing extender affected the quality and longevity of frozen-thawed Black Bengal buck semen. The freezing medium containing 10% glycerol sufficiently protected goat spermatozoa against cryoinjury during freezing and thawing, and provided an acceptable pregnancy rate. Further examination of other factors that interact and influence the spermatozoa quality following freezing and thawing remains to be studied.

Acknowledgements

Nitira Anakkul is a Ph.D. student under the Royal Golden Jubilee (RGJ) Ph.D. Program, Thailand Research Fund. The authors are grateful to Dr. Em-on Olanratmanee, Faculty of Veterinary Science, Chulalongkorn University for advice in statistical analysis. This work was financially supported by the Chulalongkorn University Centenary Academic Development Project. The CASA evaluation was kindly provided by the Zoological Park Organization, Thailand.

References

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

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

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

Afroz S, Islam MdR, Khandoker MAMY and Akter QS 2008. Cryopreservation of Black Bengal buck semen: Effects of diluents and freezing on sperm motility and morphology. Anim Sci J. 79: 550-553.

Amann RP 1999. Cryopreservation of sperm. In: Encyclopedia of Reproduction. E Knobil and JD Neill (eds) Burlington, MA: Academic Press: 773-783.

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

Apu AS, Husain SS, Khandoker MAMY, Rahman AHMS and Notter DR 2008. Investigation on seminal attributes and fertility of Black Bengal goat. Bang J Anim Sci. 37: 17-24.

Apu AS, Yahia Khandoker MAM, Husain SS, Fakruzzaman M and Notter DR 2012. A comparative study of fresh and frozen-thawed semen quality in relation to fertility of black Bengal goats. Iranian J App Anim Sci. 2: 157-161.

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

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

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

Chang YC, Jung GK, Sang RC, Dong SS, Young KK, Balasubramanian S, Sang YC and Gyu JR 2006. Influence of seasons, extenders, slow and rapid freezing on seminal characters in Korean native bucks. Reprod Dom Anim. 41: 55-60.

Deka BC and Rao AR 1986. Effect of glycerol level in Tris-based extender and equilibration period on quality of frozen goat semen. Theriogenology 26: 231-238.

Deka BB and Rao AR 1987. Effect of extenders and thawing methods on post-thawing preservation of goat semen. Indian Vet J. 64: 591-594.

Fahy GM 2010. Cryoprotectant toxicity neutralization. Cryobiology 60: S45-S53.

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

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

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

Husain SS, Horst P and Islam ABMM 1996. Study on the growth performance of black Bengal goats in different periods. Small Ruminant Res. 21: 165-171.

Khan RA 1999. A quantitative study on semen characteristics of black Bengal buck. MS Thesis, Bangladesh Agricultural University, Mymensingh.

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

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

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

Martinez-Rojero RD, Hernández J, Reyna L and Santos R. 2007. Intrauterine insemination with frozen-thawed semen in Creole goats, synchronized in estrous during the nonbreeding season. Res J Anim Sci. 3: 76-80.

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

Medeiros CMO, Forell F, Oliveira ATD and Rodrigues JL 2002. Current status of sperm cryopreservation: Why isn't it better? Theriogenology 57: 327-344.

Mobini S, Heath AM and Pugh DG 2002. Theriogenology of sheep and goats. In: Sheep and Goat Medicine. 1st ed. DG Pugh (ed). WB Saunders Company: 129-186.

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

Mortimer ST 2000. CASA-practical aspects. J Androl. 21: 515-524.

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

Peterson K, Kappen MAPM, Ursem PJF, Nöthling JO, Colenbrander B and Gadella BM 2007. Microscopic and flow cytometric semen assessment of Dutch AI-bucks: Effect of semen processing procedures and their correlation to fertility. Theriogenology 67: 863-871.

Purdy PH 2006. A review on goat sperm cryopreservation. Small Ruminant Res. 63: 215-225.

Ritar AJ, Ball PD and O'May PJ 1990. Examination of methods for the deep freezing of goat semen. Reprod Fertil Dev. 2: 27-34.

Silva AR, Cardoso RCS, Uchoa C and Silva LDM. 2002. Effect of Tris-buffer, egg yolk and glycerol on canine semen freezing. The Vet J. 164: 244-246.

Singh MP, Sinha AK and Singh BK 1995. Effect of cryoprotectants on certain seminal attributes

and on the fertility of buck spermatozoa. Theriogenology 43: 1047-l053.

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

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

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

Taggart DA, Leigh CM, Steele VR, Breed WG, Temple-Smith PD and Phelan J 1996. Effect of cooling and cryopreservation on sperm motility and morphology of several species of marsupial. Reprod Fertil Dev. 8: 673-679.

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

Tuli RK, Schmidt-Baulain R and Holtz W 1992. Computer-assisted motility assessment of spermatozoa from fresh and frozen-thawed semen of the bull, boar and goat. Theriogenology 38: 487-490.

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

Waterhouse KE, Hofmo PO, Tverdal A and Miller RRJr. 2006. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction 131: 887-894.

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