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Original Article

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Abstract

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

Keywords: cryopreservation, Equex STM paste, semen extender, goat

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บทคัดย่อ

ผลของสารลดแรงตึงผิว อีเควก เอส ที เอ็ม เพส ต่อคุณภาพและรูปแบบการเคลื่อนที่ของอสุจิในน้ำเชื้อแพะแช่แข็ง

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

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเปรียบเทียบคุณภาพของน้ำเชื้อแพะที่ผ่านการแช่แข็งโดยใช้สารละลายน้ำเชื้อที่ใส่และไม่ใส่สารลดแรงตึงผิว อีเควก เอส ที เอ็ม เพส (Equex STM Paste) ทำการรีดเก็บน้ำเชื้อจากแพะ 6 ตัว โดยน้ำเชื้อที่นำมาแช่แข็งมีคะแนนความเคลื่อนไหวมากกว่าร้อยละ 60 และมีความเข้มข้นมากกว่า 1,500 x 10^6 ตัว/มิลลิลิตร ใช้สารละลายพื้นฐานทริสและไข่แดงในการแช่แข็งน้ำเชื้อ ทำการประเมินอัตราการเคลื่อนที่ของตัวอสุจิ อัตราส่วนตัวอสุจิมีชีวิต ความผิดปกติของรูปร่างอสุจิ ความผิดปกติของอะโครโซม และความสมบูรณ์ของเยื่อหุ้มอสุจิในน้ำเชื้อสดและภายหลังการละลาย ตรวจประเมินรูปแบบการเคลื่อนที่ของอสุจิด้วยเครื่องตรวจวิเคราะห์อสุจิ (Computer-assisted-semen analysis; CASA) ภายหลังการเก็บรักษาที่ 37 องศาเซลเซียสที่ 0, 1, 2 และ 3 ชั่วโมง ผลการศึกษาพบว่ากลุ่มที่แช่แข็งด้วยสารละลายที่มีอีเควก เอส ที เอ็ม เพส มีอัตราการเคลื่อนที่สูงกว่าที่ 0 (p=0.0009), 1 (p>0.05)และ 2 ชั่วโมง (p>0.05) รวมทั้งมีอัตราส่วนอสุจิมีชีวิต (p=0.0002) หางปกติ (p<0.0001) และร้อยละของอสุจิที่มีอะโครโซมปกติ (p>0.05) สูงกว่า ส่วนความสมบูรณ์ของเยื่อหุ้มอสุจิ รูปร่างหัวปกติ และรูปแบบการเคลื่อนที่ของอสุจิในบางลักษณะมีค่าสูงแต่ไม่พบความแตกต่างอย่างมีนัยสำคัญในกลุ่มที่ไม่ใช้สารลดแรงตึงผิว สรุปได้ว่าการใส่ อีเควก เอส ที เอ็ม เพส ในสารละลายน้ำเชื้อช่วยเพิ่มอัตรารอดของตัวอสุจิแพะในช่วง 2 ชั่วโมงภายหลังการละลาย

คำสำคัญ: การแช่แข็ง อีเควก เอส ที เอ็ม เพส สารละลายน้ำเชื้อ แพะ

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Introduction

Frozen semen is normally used in artificial insemination (AI) in goat; however, freezing and thawing reduce its semen qualities. An addition of cryoprotectant(s) in a freezing medium is necessary in order to minimize both physical and chemical stresses. Glycerol and egg yolk are commonly used as cryoprotectants in goat semen freezing (Ritar and Salamon, 1982; Tuli and Holtz, 1994). Nevertherless, the diluents containing egg yolk cause detrimental effect on seminal plasma of the spermatozoa. This is due to the presence of egg yolk coagulating enzyme in the seminal plasma of goat (Iritani and Nishikawa, 1963), that why a removal of seminal plasma by centrifugation to protect the sperm cells before cooling and freezing is necessary. The study on modifications of the diluents has been investigated (Purdy, 2006).

Recently, an addition of Equex STM Paste to a Tris extender has been used to improve post thawed quality of spermatozoa in many species. It exerts a beneficial effect on sperm quality of cryopreserved semen of dog (Rota et al., 1997; Peña and Linde-Forsberg, 2000), cat (Axnér et al, 2004), pig (Pursel et al., 1978; Buranaamnuay et al., 2009), cattle (Arriola and Foote, 1987), horse (Martin et al., 1979; Jimenez, 1987), deer (Cheng et al., 2004) and sheep (Akourki et al., 2004). Addition of Equex STM Paste may also improve the freezability of goat semen.

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

The aim of this study was to evaluate the effect of Equex STM Paste supplementation in semen extenders on goat sperm quality after cryopreservation.

Materials and Methods

All chemicals used in this study were

purchased from Sigma St Louis, USA, unless otherwise specified.

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

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

Freezing and thawing of spermatozoa: Each semen sample was placed in a centrifuge tube, diluted with warm washing solution (37^{o}C), at 1:9 ratios, and followed by centrifugation at 940 x g for 10 minutes. The supernatant was discarded and the semen pellet was dispersed in the leftover medium and divided into two aliquots. They were diluted with glycerolated 10% (v/v) egg yolk Tris-citric-fructose extender with (E+) or without (E-) 1% Equex STM Paste, and the concentration was adjusted to 200 x 106 sperm/straw (800 x 10^6 sperm/ml). The diluted semen was equilibrated at 4^{o}C for 4 hours. After equilibration, the samples were loaded into 0.25 ml French mini straws (Minitüb, Landshut, Germany). The filled straws were placed horizontally approximately 4 cm above the liquid nitrogen level for 10 min in a styrofoam box, before being plunged into the liquid nitrogen.

Thawing process of frozen semen was done in a 37^{o}C water bath for 30 seconds (Deka and Rao, 1987). The post-thawed semen samples were assessed for motility, morphology, viability, acrosome integrity and membrane integrity, before processing and after freezing and thawing. Using CASA, all post-thawed samples were evaluated for motility characteristics after 0 (5 min post-thawing), 1, 2 and 3 hours incubations (37^{o}C).

Semen evaluation

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

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

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

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

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

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

CASA: The frame rate for semen analysis was set up at 45 frames at 60 Hz. The post-thawed sperm were analyzed for total motility (MOT, %), progressive

motility (PMOT, %), average path velocity (VAP, μm/s), straight velocity (VSL, μm/s), cell velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (a ratio of VSL/VAP; STR, %) and linearity (a ratio VCL/VAP; LIN, %). Each semen sample was scanned in three different selected fields, and the mean value of the three fields was used for statistical analysis.

Statistical Analysis: The results are presented as mean ± standard deviation. The differences of posted-thawed semen quality between the E+ and E- group were analyzed based on a student T-test using SAS statistical software (SAS version. 9.0 Cary, N.C., USA) at the significance level of $p<0.05$.

Table 1 The seminal characteristics of fresh and post-thawed semen with (E+) and without (E-) Equex STM Paste (mean percentage + standard deviation).

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

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

Results

The quality characteristics of fresh goat semen were in a normal range by means of total volume (1.00±0.38 ml), pH (7.22±0.25), sperm concentration (2,803.8±1,006.7x10^6 spermatozoa/ml), mass movement (3.12±0.72) and motility (71.6±6.24%). Over all, cryopreservation significantly reduced sperm quality compared to fresh spermatozoa by means of sperm plasma membrane, % normal tail sperm, acrosomal membrane integrity and also the functional membrane integrity (sHOST positive spermatozoa). Equex STM paste significantly improved post-thaw quality of goat spermatozoa in terms of the sperm viability ($p=0.0002$) and also the percentage of 'normal-tail' spermatozoa ($p<0.0001$) when compared with no-Equex freezing extender (Table 1). The number of acrosome-intact spermatozoa in E+ group was higher than that in E- group but the difference was not significant (Table 1). However, the percentage of normal head and sHOST were decreased in E- group with no significant difference (Table 1).

To confirm the positive effect of Equex supplementation on post-thaw sperm quality, the thermal resistance test evaluated by CASA was performed. The analysis indicated that the percentage of post-thawing motility (MOT and PMOT) in E+ was higher than E- group (Table 2). Considering at 0 h after thawing, MOT in E+ group were significantly higher ($p=0.0009$). At other incubation time, E+ group provided the better results in both MOT and PMOT except at the end point. In addition to sperm motility, freezing and thawing adversely affected the movement patterns of goat semen, irrespective to the presence of Equex STM Paste in the semen freezing extender. The percentage of VAP, VSL, VCL and ALH in both groups gradually decreased, whereas the BCF, STR and LIN remained unchanged over the incubation times. No significant difference in VAP, VSL, VCL, STR and LIN between E+ and E- groups was observed (Table 2). However, BCF in E- group was significantly higher than E+ group when goat semen was incubated at 0 ($p=0.0012$) and 2 hours ($p=0.0209$). Moreover, ALH was higher in E+ group at 0, 1 and 3 hours (Table 2).

Discussion

In this study, supplementation of Equex STM Paste in semen freezing extender improved the quality of frozen-thawed goat sperm, although the frozen-thawed semen quality in E+ group was still significantly lower than non-frozen sperm (fresh semen). It has become clear that suboptimal freezing induces irreversible cellular damages in particular at the levels of sperm plasma membrane (Ortman and Rodriguez-Martinez, 1994), acrosomal membrane (Jones and Martin, 1973) and sperm mitochondria (Watson, 1995), etc. As a result, pregnancy rates after transcervical insemination in goat remain poor compared to the direct deposition of frozen semen to the uterine horn via a laparoscopic insemination (Sohnrey and Holtz, 2005). For this reason, improving techniques aimed specifically at cryopreserving goat sperm without significant loss of sperm quality are sought to be important for goat breeding industry.

Our results indicated that Equex STM Paste protected the sperm against cryoinjury in terms of MOT, PMOT, sperm viability, acrosome integrity and tail morphology, which are in an agreement with previous studies in other species including pig (Pursel et al., 1978; Buranaamnuay et al., 2009), dog (Rota et al., 1997; Peña and Linde-Forsberg, 2000), cat (Axnér et al, 2004) and horse (Martin et al., 1979; Jimenez, 1987). For goat semen, beneficial effects of trehalose-egg yolk extender containing sodium dodecyl (lauryl) sulphate (SDS) were reported on acrosome integrity and motility (Aboagla and Terada, 2004[a,b]). Equex STM Paste gives similar result to SDS by improving not only acrosome integrity and motility but also viability and normal tail spermatozoa. Equex STM Paste acts as a detergent which SDS is an active compound. It improves the post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell

membranes, particularly acrosomal membranes, and to protect spermatozoa against the toxic effects of glycerol during the freeze-thaw process (Martin et al., 1979; Arriola and Foote, 1987).

In the present study, the percentage of intact acrosome spermatozoa was higher in E+ than E- group. The cryoprotective effect of Equex STM Paste on the acrosomal membrane is crucial since the presence of intact acrosome is necessary during the fertilization process and has been reported to highly correlate with fertility of frozen-thawed semen (Saacke and White, 1972). However, the beneficial effect of Equex STM Paste on intact acrosome was only observed when the egg yolk was also present. The Equex STM Paste is therefore believed to act via the modifications of egg yolk lipoproteins (Peña and Linde-Forsberg, 2000; Arriola and Foote, 1987).

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

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

Data presented as mean ± standard deviation. The different superscripts (a, b) denote values that differ statistically significant (p<0.05)

The percentage of post-thawed spermatozoa with normal membrane integrity was decreased in the extender supplemented with Equex STM Paste. Although frozen-thawed sperm in E+ group demonstrated a higher viability and motility (Table 1 and 2), the motility of these frozen-thawed spermatozoa gradually decreased over the incubation times. It seems likely that prolonged incubation of sperm in freezing extender supplemented with Equex STM Paste has a negative effect on sperm membrane/viability probably by the toxic effect of Equex STM Paste (Axnér et al., 2004). Given that the freezing extender is diluted with uterine fluid following artificial insemination, the Equex STM Paste/SDS supplementation will therefore not render the cytotoxicity in the uterus (Axnér et al., 2004).

In this study, we used CASA for evaluation of sperm motility since it has been demonstrated to be a precise and objective method in assessing motility patterns (Budworth et al., 1987; Donnelly et al., 1998; Dorado et al., 2009). For example, PMOT has become an important indicator for the sperm fertilizability because this parameter positively correlates to the pregnancy rates (Donnelly et al., 1998). Equex STM Paste enhanced the ability of spermatozoa to sustain in the incubation at temperatures close to the female body temperature for 2 h. This is similar to a report in cat which Equex STM Paste decreased the longevity of sperm during thermal resistant test (Axnér et al., 2004). However, this result is contradictory to other species such as dogs (Rota et al., 1997), cattles (Arriola and Foote, 1987), and sheep (Akourki et al., 2004).

Interestingly, it was found that an addition of Equex STM Paste did not alter the sperm motility pattern (VAP, VAC, VSL, STR and LIN) between E- and E+ groups. These motility patterns correlated with fertilizing ability of spermatozoa (Verstegen et al., 2002). However, sperm frozen with Equex had better sperm head pattern which is also believe to correlate to the pregnancy outcome. BCF and ALH were significantly higher than E- group. BCF was significantly higher than E- group when goat sperm were incubated at 0 (p=0.0012) and 2 hours (p=0.0209), while ALH was higher in E+ group (0, 1 and 3 hours). Although these motility patterns have been reported to correlate to the characteristics of sperm during fertilization (Aitken et al., 1985; Jeulin et al., 1996; Sukcharoen et al., 1996; Donnelly et al., 1998), it is still not possible to make any further conclusions that these E+ frozen-thawed sperm still retain their fertilizability in vivo until pregnancy rate after artificial insemination is examined.

Conclusion

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

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