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ประสิทธิภาพของสารละลายน้ำเชื้อชนิด Tris Egg Yolk แบบแห้งที่ถูกเก็บไว้เป็นเวลานาน ในอุณภูมิที่เย็น (4 องศาเซลเซียส) ต่อน้ำเชื้อโคแช่แข็ง

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บทคัดย่อ: สารละลายน้ำเชื้อแบบแห้งได้ผ่านการพิสูจน์แล้วว่า มีประสิทธิภาพในการเป็นสารป้องกันการแช่แข็งสำหรับอสุจิของโคที่อยู่ในน้ำเชื้อแช่แข็ง แต่ความเสถียรของสารละลายน้ำเชื้อชนิด Tris Egg Yolk แบบแห้งหลังถูกเก็บไว้เป็นเวลานาน (3 เดือน) ที่อุณหภูมิ 4 องศาเซลเซียส นั้นยังเป็นที่สงสัยอยู่ การศึกษานี้มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพสารละลายน้ำเชื้อชนิด Tris Egg Yolk แบบแห้งหลังจากถูกเก็บไว้เป็นเวลานาน (3 เดือน) ที่อุณหภูมิ 4 องศาเซลเซียส โดยนำมาผสมกับน้ำเชื้อโคแล้ววิเคราะห์คุณภาพน้ำเชื้อดังกล่าวหลังจาก การเจือจาง การบ่มปรับสมดุล และการแช่แข็ง จากนั้นทำการเปรียบเทียบกับค่าต่างๆ เหล่านั้นกับน้ำเชื้อที่ละลายอยู่ในสารละลายน้ำเชื้อสด พ่อโคนมโฮสไตน์จำนวน 8 ตัว ถูกรีดเก็บน้ำเชื้อ โดยน้ำเชื้อของแต่ละตัวจะถูกแบ่งออกเป็น 2 กลุ่ม คือกลุ่มควบคุม (n=8) และกลุ่มทดลอง (n=8) ซึ่งน้ำเชื้อในกลุ่มควบคุมจะถูกผสมด้วยสารละลายเจือจางน้ำเชื้อแบบสด และน้ำเชื้อในกลุ่มทดลองจะถูกผสมด้วยสารละลายเจือจางน้ำเชื้อสดแบบแห้ง จากนั้นทำการแช่เย็นและแช่แข็งน้ำเชื้อทั้งหมด โดยให้มีความเข้มข้นสุดท้ายของอสุจิทั้งสองกลุ่มมีค่าประมาณ $80x10^6$ ตัว/มิลลิลิตร แล้วทำการเก็บรักษาไว้ที่อุณหภูมิ -196 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง ระหว่างกระบวนการแช่เย็นและแช่แข็งจะมีการทำการวิเคราะห์ค่าร้อยละของ อสุจิที่มีการเคลื่อนที่ อสุจิที่มีการเคลื่อนที่ไปข้างหน้า อสุจิที่มีการเคลื่อนที่ไปข้างหน้าอย่างรวดเร็ว ด้วยโปรแกรม Computer Assisted Semen Analysis (CASA) และร้อยละของอสุจิที่มีความสมบูรณ์ของเยื่อหุ้มด้วยวิธี Hypoosmotic Swelling Test (HOST) ใน 3 จุดหลัก คือ หลังจาก การเจือจาง การบ่มปรับสมดุล และการแช่แข็ง ของน้ำเชื้อจากทั้งสองกลุ่ม ขั้นตอนการทดลองทั้งหมดจะถูกทำซ้ำอีก 3 ครั้ง โดยทำการตรวจวิเคราะห์คุณภาพน้ำเชื้อที่อยู่ในสารละลายน้ำเชื้อแบบแห้งที่ถูกเก็บไว้นาน 1, 2 และ 3 เดือนที่ 4 องศาเซลเซียส ในขวดสุญญากาศของกลุ่มทดลอง เปรียบเทียบกับน้ำเชื้อที่อยู่ในสารละลายน้ำเชื้อสดของกลุ่มควบคุม ผลการทดลองพบว่า ร้อยละของ อสุจิที่มีการเคลื่อนที่ อสุจิที่มีการเคลื่อนที่ไปข้างหน้า อสุจิที่มีการเคลื่อนที่ไปข้างหน้าอย่างรวดเร็ว และอสุจิที่มีความสมบูรณ์ของเยื่อหุ้มหลังจาก การเจือจาง การบ่มปรับสมดุล และการแช่แข็ง ของน้ำเชื้อจากกลุ่มควบคุมไม่แตกต่างจากค่าเหล่านั้นของน้ำเชื้อในสารละลายน้ำเชื้อสดแบบแห้ง และในสารละลายน้ำเชื้อแบบแห้งที่เก็บไว้นาน 1, 2 และ 3 เดือน ของกลุ่มทดลอง ($P>0.05$) ซึ่งค่าต่าง ๆ เหล่านี้เป็นที่ยอมรับสำหรับบ่งชี้ว่า สารละลายน้ำเชื้อแบบแห้งที่ถูกทดลองทั้งหมดนั้นสามารถเป็นสารป้องกันการแช่แข็งของอสุจิในน้ำเชื้อแช่แข็งได้ สรุปสารละลายน้ำเชื้อชนิด Tris Egg Yolk แบบแห้งที่ถูกเก็บรักษาไว้เป็นเวลานาน (3 เดือน) ยังคงมีประสิทธิภาพดีในการรักษาคุณภาพน้ำเชื้อโคแช่แข็ง

คำสำคัญ: เก็บไว้เป็นเวลานาน น้ำเชื้อโคแช่แข็ง สารป้องกันการแช่แข็ง สารละลายน้ำเชื้อชนิด Tris egg yolk แบบแห้ง CASA

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Efficiency of Long-term Storage at Chilling Temperatures (4°C) of Lyophilized Tris Egg Yolk Extender on Frozen Bovine Semen

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Abstract: The lyophilized extender was proved that it had efficiency to be cryoprotectant for spermatozoa in frozen bovine semen. But its stability after long term storage was in question. This study also was assigned to evaluate the long-term storage (3 months) of lyophilized Tris egg yolk extender at 4 ^{0}C by analysis the quality of bovine semen after dilution, equilibration and freezing and compare their parameters of semen in fresh Tris egg yolk extender. Eight Holstein bulls was semen collected that each batch of collection was divided equally into two groups. Control group (n=8) and Treatment group (n=8) were diluted with fresh extender and fresh lyophilized extender of Tris egg yolk, respectively. Optimal sperm concentration was adjusted as 80x10^6/mL. The semen diluents were further processed for cooling and freezing, after that it would be stored at -196 ℃ for 24 hours. The sperm motilities of both groups were evaluated in terms of sperm motility, progressive sperm motility, rapid sperm motility using computer assisted semen analysis (CASA) and sperm plasma membrane integrity by hypoosmotic swelling test (HOST). The semen diluents of both groups were analyzed three points such as after semen dilution, equilibration and thawing after freezing. The experiments were repeated for three more times. They were repeated of semen quality analysis that the semen in lyophilized extender after storage at 4 ^{0}C in vacuum bottles for 1 month, 2 and 3 months of treatment group were analyzed and compared to the semen in fresh extender of control group. The results showed, the percentages of sperm motility, progressive sperm motility, rapid sperm motility and HOST positive after dilution, equilibration and freezing of semen in fresh extender of control group were not significantly different from those parameters of the semen in fresh, 1-month, 2-months and 3 months storages of lyophilized extenders of treatment group (P>0.05) that the all values of spermatozoa in treatment groups were accepted for good cryoprotectant of these lyophilized extenders in frozen semen. In conclusion, the long-term storage (3 months) of lyophilized Tris egg yolk extender still had good efficiency to preserve the frozen bovine semen quality.

Keywords: CASA, Cryopreservation, Frozen bovine semen, Long-term storage, Tris egg yolk, Lyophilized extender

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Introduction

Artificial insemination (AI) is the most successful reproductive technologies in domestic animals. One of the advantages of AI expresses for rapid dispersal of greater germplasm resources to enhance cattle productivity. It widely knows that the bovine frozen semen is the most commonly used for artificial insemination (AI) in dairy and beef industries for many years. However, in frozen semen need added extender which has various elements during cooling and freezing periods. The extender provides nutrients for spermatozoa, non-toxic to spermatozoa, buffer containing, keeping capability of spermatozoa progressive motility and able to be cryoprotective agent on spermatozoa from cold shock effect.

Egg yolk is the most common active ingredient that it is added in semen extender to be sperm cryoprotectant from the harmful of cooling and freezing effects. The added egg yolk has the effective constituent of low density proteins (LDL) that able to enhance sperm motility, phospholipid sperm membrane integrity, and sperm fertilization ability during freezing-thawing procedure (Forouzanfar et al., 2010; Hu et al., 2010). Moreover, egg yolk diluent also still provides sperm protection as the antioxidative agent during storage process (Kumar et al., 2003). In addition, the solution of egg yolk base combination with Tris and cryoprotective glycerol, has long be used in bovine semen diluent to develop of effective extender to protect the extracellular membrane of spermatozoa from ice crystallization damage during cooling-freezing procedure and storage at -196 ^{0}C as a cryopreservation of frozen bovine semen. This combination yields Tris egg yolk-glycerol extender to dilute in bovine semen before cooling and deep-freezing methods, which has now come to be a standard extender (Wall and Foote, 1999). However, the fresh egg yolk base extender is unable to be long term storage. It should be freshly prepared short period before used that it is sometimes inconvenient in some cases. Thus, in recent years, the need of instant extenders is available and has arisen for conventional convenience protocol.

Lyophilization is the freeze-drying process for conservation of biological products which involve of a both freezing and dehydration of for preservation as well as blood plasma, human tissue, foods as well as drug and vaccine. This technique is able to sustain stability and viability of biological product for long term storage (Moustacas et al., 2011; Alcay et al., 2016). During the freeze-drying process is conducted at temperature below -40 ^{0}C, all or most proteins and its biological activity are preserved and still stopped as dry state of lyophilization

(Gaidhani et al., 2015). Therefore, one of the benefits of lyophilized Tris egg yolk extender has been successfully and able to storage longer (Alcay et al., 2015). It is also available, efficiency and convenient using for bovine semen cryopreservation (Kajaysri et al., 2017). However, the stability of lyophilized Tris egg yolk extender is still in question, due to several factor can affect this lyophilized extender stability. Moister and oxygen are two factors of the most important which can destabilize the lyophilized extender (Gaidhani et al., 2015). Thus, the use of packaging of lyophilized Tris egg yolk extender must be impenetrable to moister and oxygen from atmosphere. Moreover, the storage in chilling temperature such as in refrigerator (4-8 ^{0}C) of lyophilized products is able to extend their life spans (Gaidhani et al., 2015).

This study was assigned to measure the stability of long-term storage at refrigerator temperatures of lyophilized Tris egg yolk extender by considering the quality of bovine semen diluent in terms of sperm motility, progressive sperm motility, rapid sperm motility and sperm hypoosmotic swelling test (HOST) after semen dilution, equilibration and thawing after freezing, and compare their parameters to fresh Tris egg yolk extender in bovine semen diluent.

Materials and Methods

This experiment was approved by the Animal Ethics Committee of Faculty of Veterinary Medicine, Mahanakorn University of Technology (MUT). Bangkok, Thailand. All chemicals were purchased from Sigma Chemical company (St. Louis, MO, USA) unless stated otherwise.

Experimental design

Eight Holstein-Friesian bulls were semen collected by artificial vagina (AV) twice a week. Shortly after semen collection, the semen samples were kept in a water bath (37^{0}C) until use. Each collected semen from each bull was evaluated for criteria of color, consistency, wave motion, sperm concentration and percentage of sperm motility. The semen samples with normal color, thick consistency, rapid wave motion (2-4 of 0-4 scales), ≥800x10^{6}/mL of sperm concentration and ≥ 70% of sperm motility would be used in this study (Barszcz et al., 2012). After the first state of the semen quality evaluations, the all semen samples were divided equally into two groups. Group 1 (control group, n=8) was diluted with fresh extender and Group 2 (treatment group, n=8) was diluted with fresh lyophilized extender. Each extender was diluted in semen sample of each group to have the final sperm concentration approximately 80x10^{6}/mL at 37°C in water bath. Group 1 and Group 2 were

separated into three representative samples for semen evaluation at three differences period of critical points of frozen-thawed semen procedure as followed A.) after dilution: fresh semen samples just were diluted with extenders and kept at 37 ºC in water bath, B.) after equilibration: the diluted samples were filled in mini-straws likewise the frozen-thawed semen then equilibrated at 4 ºC for 4 hours in the refrigerator, and C.) after freezing-thawing: the cooling samples (4 ºC) were proceeded for frozen-thawed semen and remained in liquid nitrogen for further 24 hours. In addition, Computer Assisted Semen Analysis (CASA; IVOS program, motility analyzer version 12.3; Hamilton-Thorne, Biosciences, USA) was used for three parameters of 1.) percentages of sperm motility, 2.) progressive sperm motility and 3.) rapid sperm motility. The sperm plasma membrane integrity was done by hypoosmotic swelling test (HOST). All diluted semen would be evaluated with these parameters at 3 critical points (after dilution, after equilibration and after freezing-thawing). In addition, the semen equilibration, cooling, freezing and thawing procedures were followed by the standard methods of Lumphayaklang Livestock Semen Production Center, Department of Livestock Development, Lopburi, Thailand. These experiments were also repeated for three more times that the fresh lyophilized extender was replaced with 1, 2 and 3 months storages of lyophilized extenders, respectively.

Experimental animals

The study was conducted from March to June with the outdoor temperatures ranging from 27 to 42 ^{0}C and an average relative humidity of 75.45%. Eight healthy Holstein-Friesian bulls age 3-8 years old and free from any anatomical or reproductive disorders were randomized and semen qualities evaluated to use for this study. Their body weight was about from 800 to 1,000 kg and their body condition score was about 3.38 out of 5 according to the scale of Edmonson et al. (1989). The bulls were housed in free stall barn of Lumphayaklang Livestock Semen Production Center, Department of Livestock Development, Lopburi, Thailand. They were fed daily with concentrates containing 14.0 % crude protein and roughage (corn silage and hay) and had access to clean water *ad libitum*. Moreover, they could walk freely in a grass field in the daytime and come back to the stall barn in the evening about three times a week. The bulls were dewormed four times a year and routinely vaccinated for disease prevention following the standard vaccination program provided by the Department of Livestock Development.

Extender preparation

The extender was prepared by the same technician and divided into two groups following 1) fresh (control group) and 2) lyophilized (treatment group) extenders. The fresh extender was prepared daily prior use. In 1 liter of fresh extender contained 24.22 g Tris (Sigma, USA), 13.6 g citric acid, 10 g fructose, 1,000,000 IU penicillin G, 1 g dihydrostreptomycin, 20% (v/v) egg yolk, 8% (v/v) glycerol and 720 mL distilled water (Kajaysri et al., 2017). The lyophilized extender consisted similarly component to fresh extender but without 8% (v/v) glycerol. Afterwards, the lyophilization procedure was performed by using the freeze-drying automatic process in machine (ilShin Bio Base Freeze-Dryer, USA). Moreover, the lyophilized extender samples were packed in vacuum packaging and stored at 4 ºC in refrigerator until used. The long-term storage samples were kept for further 1, 2 and 3 months in the refrigerator. Prior use, dried extender was rehydrated with distilled water and added 8% (v/v) glycerol until reached the volume of 1 liter.

Evaluations of motility and plasma membrane integrity

The sperm motilities evaluation of three differences period of critical points of frozen-thawed semen procedure (see above) was analyzed by CASA in term of three parameters of percentages of sperm motility, progressive sperm motility, rapid sperm motility. The examination of sperm plasma membrane integrity was the one criterion which used for semen quality evaluation by sperm hypoosmotic swelling test (HOST). The HOS solution was compounded of 150 mOs/L solution of sodium citrate and fructose which was conducted according to the procedure of Jeyendran et al. (1984) and Rasul et al. (2000). Briefly, 1 mL of HOS solution of was mixed with 50 µL of semen diluent sample and incubated at 37 ^{0}C for 60 minutes. Immediate after incubation, one drop (approximately 10 µL) of the incubated semen sample was placed onto the glass slide and covered with coverslip. This slide was observed under phase contrast microscope (x400). At least 200 spermatozoa were counted randomly selected microscopic fields. Spermatozoa with swollen tail or coiled were counted as percentage of HOST positive and recorded (Jeyendran et al., 1984; Rasul et al., 2000). However, the cooling and frozen-thawed semen samples had to be warmed in warm water at 37 ºC for 30 min before used.

Statistical analyses

All parameters were analyzed using SPSS statistical package (SPSS V.10, Chicago, USA).

Results

All evaluated parameters of spermatozoa were done at the critical three points as after dilution at 37 ^{0}C, equilibration at 4 ^{0}C and deep freezing at -196 ^{0}C by using CASA and HOST. In this study, the efficiency of lyophilized extender would be examined for 4 times in each condition as a fresh lyophilization condition, 1-month, 2- and 3-months storage conditions which compared with the fresh extender. The average (Mean±SD) value of each parameter in each critical point and each condition from both groups was showed in Table 1-4. The percentages of sperm motility, progressive sperm motility, rapid sperm motility and HOST positive after dilution, equilibration and freezing of control group were not significantly different from those parameters of all four conditions of lyophilization's extenders (first = fresh lyophilized extender, second = 1-month storage, third = 2-months storage and fourth = 3-months storage) in treatment group. (P>0.05).

Table 1 Percentage (mean±SD) of semen evaluation parameters in three critical points of fresh and fresh lyophilized extenders

Parameters	Semen with fresh extender	Semen with fresh lyophilized extender
1) After dilution		
a. sperm motility	83.52±4.79	81.34±5.28
b. progressive sperm motility	58.47±4.56	56.13±5.30
c. rapid sperm motility	74.50±6.95	65.50±8.17
d. sperm HOST positive	78.48±6.76	80.87±5.75
2) After equilibration		
a. sperm motility	72.24±7.56	70.18±6.32
b. progressive sperm motility	30.28±5.80	31.45±4.35
c. rapid sperm motility	60.55±7.50	61.45±10.59
d. sperm HOST positive	60.57±5.35	55.87±8.87
3) After freezing-thawing		
a. sperm motility	43.85±7.45	41.25±7.59
b. progressive sperm motility	21.67±5.55	19.87±4.87
c. rapid sperm motility	31.78±6.48	32.10±7.78
d. sperm HOST positive	33.82±7.59	35.27±6.86

No significant differences in the same parameters and rolls between both groups (P>0.05)

Table 2 Percentage (mean±SD) of semen evaluation parameters in three critical points of fresh and 1-month lyophilized extenders

Parameters	Semen with fresh extender	Semen with 1-month lyophilized extender
1) After dilution		
a. sperm motility	83.75±5.99	79.62±4.17
b. progressive sperm motility	56.25±8.36	53.12±3.52
c. rapid sperm motility	75.88±4.73	69.62±6.56
d. sperm HOST positive	80.88±5.43	75.74±7.95
2) After equilibration		
a. sperm motility	71.37±8.89	73.37±8.03
b. progressive sperm motility	34.00±9.68	33.25±7.79
c. rapid sperm motility	63.25±13.95	64.62±9.75
d. sperm HOST positive	60.88±8.45	58.97±9.34
3) After freezing-thawing		
a. sperm motility	47.37±6.92	46.00±8.70
b. progressive sperm motility	28.00±4.10	25.00±2.87
c. rapid sperm motility	31.50±13.25	34.62±7.07
d. sperm HOST positive	27.75±5.23	24.63±6.32

No significant differences in the same parameters and rolls between both groups (P>0.05)

Discussion

From these finding results that mean the lyophilized extender was able to preserve the sperm quality in term of cryoprotectant agent similar to fresh extender on bull semen according to the previous report in bull semen (Kajaysri et al., 2017) and in ram semen (Alcay et al., 2015). In addition, this study was not assessed to the other parameters of sperm characteristics such as sperm abnormality, sperm viability and acrosome membrane integrity. However, the using of three parameters of sperm motions and one parameters of sperm plasma membrane integrity for semen evaluation at three focal points (diluting, equilibration, freezing-thawing) were suitable and practical to analyze the semen quality in different extenders after frozen semen processing. According to the previous study that the motion of spermatozoa is an important characteristic for transportation of spermatozoa into female reproductive tract and successful for fertilization with the

oocyte (Sundararaman et al., 2012). Moreover, the HOST is provided to evaluate the sperm membrane function. The principle bases on the observation of sperm morphology that it exposes in hypoosmotic solutions. The cell volume of spermatozoa will be increased and swollen with coiled tail when it is placed in hypoosmotic condition (Jeyendran et al., 1984; Rasul et al., 2000). The HOST is also useful tool to prove sperm membrane damage which is induced by cryopreservation in frozen-thawed semen process (Correa and Zavos, 1994). These both characteristics as motion and membrane integrity of spermatozoa will be reduced via the processing of frozen semen. Thus, this study found that the percentages of sperm motility, progressive sperm motility, rapid sperm motility and sperm HOST positive in mixture of semen and extender after deep freezing at -196 ^{0}C and thawing at 37 ^{0}C were also reduced in both mixtures of fresh extender and lyophilized extender. However, these characteristics of spermatozoa in 3

Table 3 Percentage (mean±SD) of semen evaluation parameters in three critical points of fresh and 2-month lyophilized extenders

Parameters	Semen with fresh extender	Semen with 2-months lyophilized extender
1) After dilution		
a. sperm motility	80.25±3.24	80.00±5.85
b. progressive sperm motility	55.00±2.97	52.00±1.99
c. rapid sperm motility	72.88±4.38	70.50±4.86
d. sperm HOST positive	76.75±7.15	71.87±8.65
2) After equilibration		
a. sperm motility	69.75±6.49	71.25±5.09
b. progressive sperm motility	30.75±6.29	31.87±4.45
c. rapid sperm motility	62.38±8.46	63.75±5.31
d. sperm HOST positive	59.55±6.43	55.85±7.16
3) After freezing-thawing		
a. sperm motility	47.50±12.38	44.50±4.34
b. progressive sperm motility	26.37±3.77	22.00±2.67
c. rapid sperm motility	36.00±11.08	36.87±4.05
d. sperm HOST positive	31.50±7.65	30.87±9.87

No significant differences in the same parameters and rolls between both groups (P>0.05)

Table 4 Percentage (mean±SD) of semen evaluation parameters in three critical points of fresh and 3-month lyophilized extenders

Parameters	Semen with fresh extender	Semen with 3-months lyophilized extender
1) After dilution		
a. sperm motility	86.00±6.55	81.25±6.11
b. progressive sperm motility	53.50±9.13	50.62±6.11
c. rapid sperm motility	77.00±10.98	76.87±7.70
d. sperm HOST positive	80.35±13.59	72.88±12.57
2) After equilibration		
a. sperm motility	70.13±9.96	68.38±7.70
b. progressive sperm motility	37.38±7.55	33.00±4.80
c. rapid sperm motility	51.13±14.21	45.88±7.70
d. sperm HOST positive	50.16±8.84	45.87±10.55
3) After freezing-thawing		
a. sperm motility	54.25±14.21	49.38±7.80
b. progressive sperm motility	36.62±12.79	30.87±10.04
c. rapid sperm motility	44.87±14.81	41.5±8.54
d. sperm HOST positive	28.87±14.73	29.62±8.92

No significant differences in the same parameters and rolls between both groups (P>0.05)

months storage of lyophilized extender were still similar to those characteristics of spermatozoa in fresh extender. For the finding result in this study could be interpreted that the 3 months lyophilized extender had still efficiency to be cryoprotectant as well. However, the long-term storage lyophilized extender could be decreased this efficiency when it was kept in unreal vacuum or poor sealing packaging due to the moister and oxygen from atmosphere were able to remain or impenetrate into the packaging and oxidize with long term storage lyophilized extender according to Gaidhani et al. (2015). In this study used the 100 mL glass bottle with rubber closer and paraffin sealing for lyophilized extender keeping. After that the needle and syringe were applied for air suction from the bottle. This extender was also kept in vacuum bottle that the moister and oxygen from atmosphere was unable to impenetrate into the extender. Then, the lyophilized extender with long term storage for 3-months also still had the stability and

efficiency to be cryoprotectant of frozen semen as well. However, it was difficult to make sure of the real vacuum bottle for this study, especially at the space of the bottle's neck which might be possible to remain the oxygen and moisture inside and cause of instability of the more than 3 months storage lyophilized extender.

Moreover, the 3 months lyophilized extender could still provide the acceptable values for preservation of the frozen semen quality after freezing and thawing that the values of sperm motion characteristics were similar to those standard values of the CASA instruction belonging the Lumphayaklang Livestock Semen Production Center. Moreover, its value of sperm HOST positive was also similar to the standard value of bovine semen (Chaudhary et al., 2017). Regarding to the results, the long-term storage lyophilized extender had efficiency for cryoprotectant of spermatozoa in frozen semen process and it was used conveniently more than the fresh extender using due to the lyophilized extender could be long-term storage (3 months) and unnecessary for its total preparing daily prior use like fresh extender. Therefore, the lyophilized extender might be able to use instead the fresh extender in near future as well. In addition, for the expanding study should also examine the efficiency of long-term storage lyophilized extender with bovine semen diluent for AI in bovine female in further.

It could be concluded that the long-term storage (3 months) lyophilized extender had efficiency for cryopreservation of frozen bovine semen quality as well. It might be able to still stable for long time when it was kept in real vacuum packaging at 4 ^{0}C.

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