The Effect of adding L-Cysteine in Modified Kenny's extender on the quality of chilled stallion semen at 5°C

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Abstract

The aim of this study was to investigate the effect of supplementation of L-Cysteine in Modified Kenny's extender on chilled stallion semen quality at 5°C. Semen samples were collected from 3 stallions by artificial vagina and centrifugation to remove seminal plasma and diluted with Modified Kenny's extender to 50 x 10^6 sperm/ml. Semen samples (n=10 ejaculates) were divided into 3 portions, the first portion as the control (KE), the second portion was added with L-Cysteine 200 μmol/l (KEC200) and added L-Cysteine 400 μmol/l in the last portion (KEC400). In all samples, the percentage of progressive motility, viability and acrosome integrity were evaluated at 0, 24, 48 and 72 h after storage at 5°C. There were a significant ($P < 0.05$) lower percentage of dead spermatozoa in group KE200 than other groups at 24 h. There was a significantly lower percentage of progressive motility in KEC 400 compared toother groups at 48 h ($P < 0.05$). In addition, there was a tendency ($P = 0.1$) of lower percentage of dead spermatozoa in group KE200 than other groups at 48 h. At 72 h, comparing all groups, a higher percentage of viability in groups KEC200 and KEC400 was found, but not statistically significant. In conclusion, Addition of L-cysteine at a concentration of 200 μmol/l to the Modified Kenney's extender tend to improve the stallion chilled semen progressive motility, dead/alive, viability and acrosome integrity during chilled storage at 5°C for 48 h.

Keywords: Stallion, Sperm, Chilled semen, L-Cysteine, Antioxidant

ผลของการใส่สารแอลซีสเทอีน (L-Cysteine) ในสารละลายเจือจางน้ำเชื้อ Modified Kenney's ในการเก็บรักษาน้ำเชื้อพ่อม้าด้วยการแช่เย็นที่อุณหภูมิ 5 องศาเซลเซียส

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบผลของการเติมสารแอลซีสเทอีนในสารละลายเจือจางน้ำเชื้อ Modified Kenney's ต่อคุณภาพน้ำเชื้อพ่อม้าในการเก็บรักษาด้วยการแช่เย็นที่อุณหภูมิ 5 องศาเซลเซียส ตัวอย่างน้ำเชื้อทั้งหมด 10 ตัวอย่าง จากการรีดพ่อม้า 3 ตัว ด้วยช่องคลอดเทียม จะถูกนำมาทำการปั่นแยกน้ำเลี้ยงอสุจิออกและเจือจางด้วยสารละลายเจือจางน้ำเชื้อ Modified Kenney's ให้มีความเข้มข้น $50x10^6$ ตัว/มล. จากนั้นแบ่งเป็น 3กลุ่ม กลุ่มควบคุม (KE) กลุ่มที่เติมสารแอลซีสเทอีน ความเข้มข้น 200 ไมโครโมล/ลิตร (KE200) และกลุ่มที่เติมสารแอลซีสเทอีน ความเข้มข้น 400 ไมโครโมล/ลิตร (KE400) และเก็บรักษาที่อุณหภูมิ 5 องศาเซลเซียส ทำการตรวจเปอร์เซ็นต์การเคลื่อนที่ไปข้างหน้าของอสุจิ อัตราตัวเป็นตัวตายเปอร์เซ็นต์ตัวอสุจิที่มีชีวิต และตรวจความสมบูรณ์ของอะโครโซมที่เวลา 0 24 48 และ 72 ชั่วโมง จากการศึกษาพบว่าที่ 24ชั่วโมง กลุ่ม KE200 มีอัตราตัวตายน้อยกว่ากลุ่มอื่นๆ อย่างมีนัยสำคัญ ($P < 0.05$) ที่ 48 ชั่วโมง กลุ่ม KEC400 มีเปอร์เซ็นต์การเคลื่อนที่ไปข้างหน้าของอสุจิต่ำกว่ากลุ่มอื่นๆ อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) นอกจากนี้ยังมีแนวโน้มของอัตราตัวตายในกลุ่ม KE200 น้อยกว่ากลุ่มอื่นๆ อย่างมีนัยสำคัญ ($P = 0.1$) ส่วนที่ 72 ชั่วโมง เมื่อเปรียบเทียบผลการทดลองของทุกกลุ่ม พบว่ากลุ่ม KE200 และ KEC400 มีเปอร์เซ็นต์ตัวอสุจิที่มีชีวิตสูงกว่ากลุ่มอื่นๆ แต่ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ จากผลการทดลองสามารถสรุปได้ว่าการใส่สารแอลซีสเทอีนที่ความเข้มข้น 200 ไมโครโมล/ลิตร ในสารละลายเจือจางน้ำเชื้อ Modified Kenney's มีแนวโน้มในการเพิ่มเปอร์เซ็นต์การเคลื่อนที่ไปข้างหน้าของอสุจิ อัตราการรอดชีวิตและความสมบูรณ์ของอะโครโซม ของน้ำเชื้อพ่อม้าในการเก็บรักษาด้วยการแช่เย็นที่อุณหภูมิ 5 องศาเซลเซียส

คำสำคัญ : พ่อม้าอสุจิ น้ำเชื้อแช่เย็น สารแอลซีสเทอีน สารต้านอนุมูลอิสระ

Introduction

Over the past 10 years, technology for cooling semen has been developed. Stallion semen is highly sensitive to cold shock during cryopreservation, there by resulted in an increased usage of chilled semen (Aurich et al., 1997; Vidament et al., 1997). However, chilled semen technology will be a great interest when one is able to keep semen fertile for 1-3 days to allow transport over the regions. In fact, the longer the fertility potential of chilled semen can be extended, the easier it should be for breeders to perform artificial insemination. To do this, a proper semen extender during chilled storage is needed. It is not possible to preserve stallion spermatozoa fertility for a long time without the use of semen extender, which have several benefits to sperm such as enabling an metabolically and physiologically favorable environment to the survival of sperm cells, protecting them from unfavorable environmental conditions, for example cold shock, negative effects of seminal plasma, toxic products produced by spermatozoa and an excessive bacterial growth. Several milk and egg yolk protein components have been shown to protect spermatozoa from negative effects of low temperatures (Watson 1990; Batellier et al., 1997). Skim milk-based extenders add to glucose and antibiotics, and eventually added to other sugars and salts, are today used world wide (Kenney et al., 1975; Palmer 1984; Katila et al., 1997). They are inexpensive, easy to make, freezable and have produced good results in practice. Generally, good extender should have osmotic pressure compatible with the semen and a pH level close to neutral. The osmolality of stallion semen plasma has approximately 300 mOsm (Pickett et al., 1976). It has been reported that base on the percentage of progressive motility after 12 hours of storage, an osmolality of 350 mOsm appears to be optimal when semen is stored at 5°C in milk base extender (Varner 1991). One of the commonly use stallion semen extenders is Kenney's extender which originally introduced as part of minimal contamination techniques to prevent infection at the time of breeding (Kenney 1975).

Recently, it has been reported in many species that L-cysteine, a precursor of intracellular glutathione and taurine (Stegink 1986), with its antioxidant activity is able to prevent peroxidative damage to sperm plasma membrane during chilled storage (Gressier et al., 1994; Baker 1996; Bilodeau et al., 2001; Funahashi and Sano, 2005; Michael et al., 2007; Kledmanee et al., 2013). However, no study has been reported on the usage of L-cysteine supplementation in Kenney's extender on the quality of chilled stallion semen. Therefore, the purpose of this study was to examine the optimal concentration of L-cysteine on the progressive motility, plasma membrane integrity, and acrosome integrity of stallion semen diluted in the modified Kenney's extender during storage at 5°C.

Materials and Methods

The research proposal of this project was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC)-Mahidol University.

Animals

Three fertile stallions (Thoroughbred, Holstein, Standardbred), an average age of 9 years old, Thoroughbred, Holstein, Standardbred were used in this study. The stallions were active breeding sires and were routinely used for artificial insemination programme.

Semen extender preparation

Stallion semen extender used in this study was non-fat dry milk glucose based extender, namely Modified Kenney's extender which composed of Glucose monohydrate 49 g/L, non-fat dry skim milk 24 g/L, NaHCO38.4% and Penicillin G sodium 150,000 IU/L (Kenney et al., 1975).

Semen collection and examination

Semen samples (n=10) were collected via Colorado artificial vagina (Tharasanit et al., 2007). Collected semen was filter through gauze and then examined for basic semen characteristics included volume, color, pH, odor, sperm motility and concentration. Neat semen samples were then diluted with modified Kenney's extender in a 2:1 ratio (extender: semen) at 37°C and transport to laboratory via mobile refrigerator at 5°C.

Semen preparation and supplementation of L-cysteine

At the semen laboratory, the semen was centrifuged at 460 g for 10 min at 5°C to remove seminal plasma (HettichRotanta 460R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Each ejaculate was extended with glucose skim milk semen extender solution to obtain a final sperm concentration of 50 x 10^6 sperm/mL and divided into three equal portions and allocated into 3 groups, control group (KE) contained only Kenney's glucose skim milk semen extender, while the other two treatment groups (KEC200 and KEC400) were added with L-cysteine at a concentration of 200 and 400 μmol/l, respectively.

All diluted semen samples were stored at 5°C in a 50 ml centrifuge tube. Thereafter, the semen were divided for evaluation of progressive motility under phase contrast microscope (Olympus CX31, New York, USA), dead/alive by eosin-nigrosin staining, at 0, 24, 48 and 72 hour after storage, and examination for sperm viability by SYBR-14/EthD-1 staining and acrosome integrity by FITC/PNA staining under fluorescence microscope (Carl Zeiss, Inc., Axioskop 40, Germany) at 0 and 72 hour after storage.

Sperm progressive motility

Subjective sperm progressive motility was evaluated at 37 °C under phase contrast microscope at 100x and 400x magnification (Berger et al., 1985). Motility examinations for all semen samples were assessed by the same person. Motility was expressed as the percentage of motile sperm cells.

Sperm viability

The percentages of sperm viability were determined by 2 methods.

I. Eosin-nigrosin staining (Kaeoket et al., 2008; 2011)

The semen sample (50 μL) were mixed well with a drop of eosin-nigrosin dyes (FlukaChemie GmbH, Sigma-Aldrich, Switzerland), and the mixture (10 μL) was smeared and dried on a glass slide 1000x magnification Spermatozoa with an unstained head were regarded as live spermatozoa.

II. SYBR-14/EthD-1 (Chanapiwat et al., 2009; 2012)

Mix 70 μl diluted semen with 70 μl of TM. Then, use 50 μl mixtures mix with 3.5 μl SYBR-14. The next steps, use 10 μl mixture from last step mix with 10 μl of Ethidiumhomodimer-1, incubate at 37°C for 15 minutes. Evaluation was undertaken by counting 200 spermatozoa under fluorescence microscope at 400x magnification (Carl Zeiss, Inc., Axioskop 40, Germany). Spermatozoa with stained green with SYBR-14 were regarded as live spermatozoa, spermatozoa with stained green-red were regarded as damaged spermatozoa and spermatozoa with stained red were regarded as dead spermatozoa. The results werereported as the percentage of live spermatozoa.

Sperm acrosome integrity (Kaeoket et al., 2010)

The integrity of sperm acrosome was evaluated with fluorescein isothiocyanate-labeled peanut (Arachishypogaea) agglutinin (FITC-PNA) staining, mix 20 μl diluted semen with 10 μl of Ethidiumhomodimer-1, incubate at 37°C for 15 minutes. Then, smear 5 μl mixtures on glass slide and air dry. Fix with 96% ethanol for 30 second and air dry. The next step, spread 50 μl Fit C-PNA (dilute Fit C-PNA with PBS 1:10v/v) over the slide and incubate in a moist-dark chamber in

fridge for 30 minutes. After that, rinse with cold PBS and air dry. Evaluation was undertaken by counting 100 spermatozoa under fluorescence microscope at 1000X magnification and classified to intact acrosome, damaged acrosome and dead sperm. The results will be scored as the percentage of intact acrosome spermatozoa.

Statistical analysis

Statistical comparisons were performed using the SPSS/PC+ statistics package (version 18.0 for Windows, SPSS Inc, Chicago, IL, USA). The percentage of progressive motility, dead sperm, viability and acrosome integrity were modeled according to Randomized Complete Block Design (RCBD). Data were expressed as the mean ± standard deviation analyzed by general linear model. Probability threshold set at 5% ($P<0.05$) was considered as a significant difference.

Results

Progressive motility

There was no significant difference in progressive motility among groups at 0, 24 and 72 h (Table.1). However, there was a significant ($P < 0.05$) lower percentage of progressive motility in group KE400 than other groups at 48 h. After 48 h of storage, a dramatically decreased in progressive motility was found in all groups.

Table 1 The means percentage of progressive motility of stallion spermatozoa at different times of storage at 5°C (n=10)

Time (hour)	KE	KEC200	KEC400	pool SEM	*P*-value
0	63.00	63.00	63.50	1.63	0.855
24	60.00	55.00	52.50	2.63	0.156
48	50.00[a]	49.50[a]	41.00[b]	2.39	0.026
72	38.50	38.00	31.50	3.09	0.143

KE = Modified Kenney's extenders, KEC200 = Modified Kenney's extenders with L-cysteine 200 μmol/l and KEC400 = Modified Kenney's extenders with L-cysteine 400 μmol/l. Significant differences ($P<0.05$) in line are indicated by different letters (a, b)

Dead/alive and Viability

For the dead/alive, there was no significant difference among groups at 0 and 72 h after storage. However, there were a significant (P < 0.05) lower percentage of dead spermatozoa in group KE200 than other groupsat 24 h (Table 2). In addition, there was a tendency (P = 0.1) of lower percentage of dead spermatozoa in group KE200 than other groups at 48 h. At 72 h of storage, a dramatically increased (greater than 50%) in the percentage of dead spermatozoa was found in all groups.

Table 2 The means percentage of dead stallion spermatozoa at different times of storage at 5°C (n=10)

Time (hour)	KE	KEC200	KEC400	pool SEM	*P*-value
0	42.45	41.50	46.75	3.72	0.469
24	40.35^{ab}	7.50^{b}	45.20^{a}	2.30	0.048
48	42.15	39.40	43.35	1.93	0.122
72	55.25	54.90	56.50	2.19	0.699

KE = Modified Kenney's extenders, KEC200 = Modified Kenney's extenders with L-cysteine 200 μmol/l and KEC400 = Modified Kenney's extenders with L-cysteine 400 μmol/l. Significant differences (P<0.05) in line are indicated by different letters (a, b)

For the viability, the percentage of viability of spermatozoa during storage was higher than 50%. At 72 h, comparing all groups, a higher percentage of viability in group KEC200 was found, but not statistically significant (Table 3).

Table 3 The means percentage of viability of stallion at different times of storage at 5°C (n=10)

	Time (hour)	KE	KEC200	KEC400	pool SEM	*P*-value
Alive	0	62.80	62.51	62.20	2.91	0.99
	72	54.12	56.38	54.02	3.40	0.86
Non-alive	0	37.20	37.49	37.80	2.91	0.99
	72	44.92	43.70	46.02	3.57	0.90

KE = Modified Kenney's extenders, KEC200 = Modified Kenney's extenders with L-cysteine 200 μmol/l and KEC400 = Modified Kenney's extenders with L-cysteine 400 μmol/l

Acrosome Integrity

The percentage of acrosome integrity during storage was higher than 85%. At 72 h, comparing all groups, a higher percentage of viability in groups KEC200 and KEC400 was found, but not statistically significant (Table 4).

Table 4 The means percentage of acrosome integrity of stallion spermatozoa at different times of storage at 5°C (n=10)

	Time (hour)	KE	KEC200	KEC400	pool SEM	*P*-value
Intact	0	90.10	92.42	89.80	1.49	0.42
	72	86.79	88.69	88.79	1.64	0.63
Non-intact	0	9.90	7.58	10.20	1.49	0.42
	72	13.21	11.31	11.21	1.64	0.63

KE = Modified Kenney's extenders, KEC200 = Modified Kenney's extenders with L-cysteine 200 μmol/l and KEC400 = Modified Kenney's extenders with L-cysteine 400 μmol/l

Discussion

It is well documented that Kenney's extender is a common stallion semen extender for chilled storage at 5°C. In this study, it was revealed that supplementation of L-cysteine at a concentration of 200 μmol/l to the Modified Kenney's extender tend to improve the stallion chilled semen qualities in term of progressive motility, dead/alive, viability and acrosome integrity during chilled storage at 5°C for 48 h which is comparable to the results reported by Tharasanit et al. (2007); and superior than the study in Thai native crossbred by adding L-glutamine in stallion semen extender (Phetudomsinsuk et al., 2008). These resultsare also in agreement with the study in boar semen by Funahashi and Sano (2005) in that L-cysteine, with its antioxidant activity is able to prevent peroxidative damage to sperm plasma membrane during chilled storage for as long as 29 days. Another explanation for the superior semen qualities found in L-cysteine treated group, might be that the capacity of L-cysteine in decreasing amount of ROS and inhibiting lipid peroxidation during cold storage (Bansal and Bilaspuri, 2011) and its critical role as membrane stabilizer and capacitation inhibitor for spermatozoa (Johnson et al., 2000). Besides the reported for chilled storage semen, L-cysteine has also been reported as an antioxidant of choice for cryopreservation of semen in many species such as canine (Michael et al., 2007), bull (Bilodeau et al., 2001) and boar (Chanapiwat et al., 2009; Kaeoket et al., 2010). The underlying mechanism might be that L-cysteine has the ability to pass into cells rapidly, and be transformed to taurine, which, combined with a fatty acid in the plasma membrane, is transformed to acyl-taurine, which can improve surfactant properties and osmoregulation of the sperm membrane (Vazquez and Roldan, 1997; Esteves et al., 2007). Furthermore, it has been elucidated that a protein, namely Cysteine-Rich Secretory Protein 3 (CRISP-3), has been found to be localized in male reproductive tract in human (Udby et al., 2005), stallion (Töpfer-Petersen et al., 2005), and also in boar (Vadnais et al., 2008) and are thought to be implicated in the process of reproduction from spermatogenesis, sperm maturation,

capacitation to oocyte-sperm fusion and possibly sperm penetration of the zonapellucida (Cohen et al., 2007). This also may explain the beneficial effect of addition of L-cysteine into Kenney's extender in the present study. However, it is worth noting that the negative effect of a high dose (400 μmol/l) of L-cysteine on stallion semen qualities can be found in the present results which are in accordance with the earlier study in frozen boar semen by Kaeoket et al. (2008; 2010) who reported that spermatozoa have limiting uptake of antioxidants into their plasma membrane from external milieu.

Conclusion

Addition of L-cysteine at a concentration of 200 μmol/l to the Modified Kenney's extender tend to improve the stallion chilled semen progressive motility, dead/alive, viability and acrosome integrity during chilled storage at 5°C for 48 h.

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