The Quality of Stallion Semen in Skim Milk and Dimitropoulos Extenders Preserved at 5 °C and Ambient Temperature Supplemented with Different Sugar

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ABSTRACT

This study was conducted to evaluate the effects of sugars supplementation in skim milk based (SM) and dimitropoulos (DV) extenders on the sperm motility and viability in stallion semen storage at 5 °C and ambient temperature (24-29 °C). Semen samples were collected from 3 stallions; evaluate individually and each of them divided into 8 aliquots. Four out of eight aliquots were diluted 1:1 with SM, while the remaining four were diluted 1:1 with DV; all were then centrifuged at 1006 g (3000 RPM) for 15 min. The supernatants were discarded, and each pellet was re-diluted with SM (control), SM trehalose (SMT), SM-raffinose (SMR), SM-fructose (SMF), DV (control), DV-trehalose (DVT), DV-raffinose (DVR), and DV-fructose (DVF). The diluted semen were divided into 2 aliquots and stored at 5 °C or ambient temperature. The sperm motility and viability were evaluated every 3 h on chilled semen stored at ambient temperature, and every 12 h on those stored at 5 °C. Results of the experiments demonstrated that sperm motility and viability in DV extender significantly higher (P<0.05) in both temperature. The supplementation of fructose was the best on the motility and viability of the sperm at both temperatures compared to trehalose, raffinose, or the control group. The best extender and sugar combination was DVF, which the total motile sperm stored at 5 °C for 96 h was 45.1% followed by DVT (40.2%) and DVR (39.2%). The sperm motility in DV and SMF were 35.3% and 35.6%, respectively; these were higher than those diluted with control (28.9%); SMT (30.3%), and SMR (29.6%). The study concluded that the supplementation of fructose in DV extender (DVF) was the best combination to preserve stallion sperm motility and viability stored at 5 °C or ambient temperature.

Key words: skim milk, dimitropoulos, sugars, chilled semen, stallion


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ngencer DV dan SMF masing-masing adalah 35,3% dan 35,6%, lebih tinggi daripada yang diencerkan dengan SM kontrol (28,9%), SMT (30,3%), dan SMR (29,6%). Penelitian ini menyimpulkan bahwa suplementasi fruktosa dalam extender DV (DVF) adalah kombinasi terbaik untuk preservasi semen cair kuda baik pada suhu 5 °C maupun suhu ruangan.

Kata kunci: susu skim, dimitropoulos, gula, semen cair, kuda

INTRODUCTION

Artificial insemination is an important tool in animal production. However, cooling and cryopreservation are known to induce non-regulated capacitation-like sperm modification in numerous domestic species (Bailey et al., 2000). Storage of chilled semen at 5 °C induces a transition on sperm plasma membrane from the chilled crystalline to the gel phase. At body temperature sperm metabolism is maximal, while at ambient temperature (24-29 °C) it is lower. For each 10 °C temperature decrease, cellular metabolism is reduced by 50%; at 5 °C sperm metabolic activity is only 10% of what it would had been at body temperature (McKinnon, 1999). In the reduced metabolic state, sperm need energy to maintain their cellular activity.

Equine semen contains high sodium chloride concentration; this alters the osmotic pressure during cooling and freezing. To avoid this, the seminal plasma needs to be separated by centrifugation (Kopt el al., 2005; Morrell et al., 2009). Consequently, not only the sodium chloride, but other components such as fructose, sorbitol, enzyme and other energy sources are wasted. Besides the needs of energy (McKinnon, 1999), equine chilled semen stored in low temperature needs to be considered of sperm cold shock, which may alters the configuration of their plasma membrane (Ball, 2008; Neild et al., 2005). The increased use of cooled semen in the equine industry has consequence to the need of suitable diluents availability.

The composition of extenders varies enormously, but they are normally based upon milk or egg yolk products plus antibiotics. Nutrients, buffer solution to neutralize metabolic waste of sperm and anti cold shock are the important factors to maintain the viability of sperm during storage at low temperature (McKinnon, 1999). Sugars serve as the nutrient for sperm. The most commonly used sugar to preserve and cryo-preserve equine semen is glucose (Bozkurt et al., 2007; Pojprasath et al., 2011). Fructose is easier than glucose to be metabolized by sperm, and is commonly included in chilled and frozen semen extenders in different species (Aboagla & Terada, 2004a; Silva et al., 2005; Akhter et al., 2010). Trehalose and raffinose are extracellular cryoprotectants which stabilize plasma membrane; both of these are commonly supplemented in frozen semen extenders (Hu et al., 2010; Pojprasath et al., 2011; Slanina et al., 2012). Sperm plasma membrane damage not only occurs during freezing, but also during cooling (Aurich, 2005) refers chilling injury. Trehalose and raffinose in semen extenders are expected to reduce cold shock of sperm during storage at 5 °C. This study aims to study the effects of fructose, trehalose, and raffinose in each SM and Dimitropoulos (DV) extenders in maintaining the quality of equine chilled semen stored in ambient temperature and 5 °C.

MATERIALS AND METHODS

Animals

Three stallions (5–8 yr old) owned by Athena stable, Cinere-Depok, West Java, Indonesia used as semen source in the study. The stallions were a fourth generation (G4) of Thoroughbred, an American pinto, and a Swedish warm blood, each was in a good health and demonstrated the best quality on the daily sperm output.

Extenders

All reagents were obtained from Merck, KgaA (Darmstadt Germany) unless otherwise indicated. Extenders used in this study were skim milk (SM) based extender (Kenney et al., 1975) and Dimitropoulos extender (Ijaz & Durcarme, 1999). SM was composed of 2.4 g SM and 4 g glucose, diluted in 100 mL water. Extenders were heated to reach 92-95 °C for 10 min, allowed to cool, filtered, and then added with 100 mg streptomycin and penicillin 100,000 units.

Dimitropoulos (DV)-based extender consisted of two parts, A and B solutions. A solution was composed of 2 g glucose and 2 g fructose diluted in 100 mL water, heated in a water heater for 15 min until it reached 95 °C, allowed to cool, and stored at 5 °C for a maximum of 1 wk. B solution was composed of 2 g sodium citrate, 0.94 g glycine and 0.35 g sulphanylamide diluted in 100 mL water. The mixed solution was heated to 100 °C, stored in ambient temperature for a maximum of 1 wk.

DV-based extender was a mixed of 30% A solution, 50% of B solution, and 20% of egg yolk; the mixed solution was centrifuged at 1200 x g, for 20 min. The supernatant was collected and added with 1000 unit penicillin and 1 mg streptomycin per mL solution. Extenders semen in this study were SM or DV-based extenders (control groups) and SM or DV-based extenders supplemented with either 50 mM trehalose (Sigma, Chemical Co, St. Louis, Mo, USA), 50 mM raffinose (BDH Chemical Ltd, Poole England), or 100 mM fructose.

Semen Collection and Processing

An ejaculate from each stallion was collected twice a week, using Nishikawa type artificial vagina which was
modified with semen collecting tube of Missouri type (Nasco, Fort Atkinson, WI). On the top of the semen collecting tube was covered with gauze to strain out the gel fraction of the ejaculates (Arifiantini et al., 2006).

Immediately after collection, semen sample was assessed for volume, color, consistency, pH, motile and viable sperm, sperm concentration, and sperm morphology. Sperm concentration was measured by counting chamber, using a dilution of 1:100 with 3% saline solution (Baracaldo et al., 2007). Sperm morphology was examined by carbofuchsin eosin method (Arifiantini et al., 2010; Arifiantini, 2012).

Only ejaculate with concentration greater than 150.10^6 sperm/mL having >65% progressively motile and less than 30% sperm abnormality selected for preservative. Chilled semen of each sample was prepared by dividing the semen equally into eight tubes; semen samples in the first four tubes were diluted 1:1 with SM based extender; semen in the remaining four tubes were diluted 1:1 with DV-based extender. All semen samples in tubes were centrifuged (Hettich, EBA 3S, Tuttlingen, Jerman) for 15 min at 1006 x g (3000 RPM), and then the supernatant was discarded. The pellet (sperm) in four tubes added with SM was each mixed with SM (control), trehalose (SMT), raffinose (SMR), or fructose (SMF). Similarly, the pellet in the remaining four tubes added with DV-based extender was each mixed with DV (control), trehalose (DVT), raffinose (DVR), or fructose (DVF). Final sperm concentration in each solution was 200 x 10^6 sperm mL^-1. All extended semen then divided into two tubes and stored at 5 °C and ambient temperature.

Assessment of Chilled Semen Quality

The assessment conducted every 3 h on each chilled semen stored at ambient temperature, and 12 h on those stored at 5 °C. Sperm motile was assessed subjectively under a light microscope (Olympus CH20, with 400x magnification) equipped with a heating stage adjusted to +37 °C. Motility estimations were performed from 5 different fields in each sample by the same person throughout the study.

The sperm viability was assessed using eosin-nigrosin solution (3.3 g eosin Y, 20 g nigrosin, 1.5 g sodium citrate in 300 mL of water, adjusted to pH 7.0) (Bart & Oko, 1989). Equal volumes of sperm suspension were mixed, 20 μL of this mixture were smeared into a clean microscope slide (pre warmed to 37 °C) and allowed to dry at heating stage (37 °C). Ten fields per slide (at least two hundred sperm) were directly counted using light microscope. Sperm colored pink were considered non-viable and unstained (clear) cells were counted as a live.

Statistical Analysis

All values were calculated as means ± standard deviation of the means (± SD), and p value < 0.05 was considered significant. Analysis used 2 x 4 factorial treatment (DV versus SM based extender; control versus fructose, trehalose and raffinose) on sperm motility and viability, with 3 stallions and 4 replication (n-12).

RESULTS AND DISCUSSION

Fresh Semen Quality

Fresh semen in the study was good quality; semen macroscopic evaluation demonstrated the mean volume without gel was 33.6±12.9 mL, pH was 7.0±0.2, thin consistency, and white-cloudy in semen color. The motile and viable sperm were 70.0±5.4% and 83.3±18.4%, respectively; sperm concentration was 213.0±21.9x10^6/mL; sperm with normal morphology was 75.2±7.0%.

The Sperm Survival of Stallion Chilled Semen Stored at 5 °C

The proportion of motile cells, in all extender progressively decreased from the 2nd day of storage to reach a minimum value at day 4 (96 h). Since the second days of storage until day 4, the highest percentage of motile sperm was found in both extender supplemented with fructose (P<0.05). No significantly difference was found between trehalose and raffinose in DV extender, while in SM extender supplementation of fructose seem to be better extender compare to trehalose, raffinose, or control (Table 1).

During storage at 5 °C, the percentage of viable sperm in DV was decreased by 3.4% to 4.3% for every 12 h of observation; while it was decreased by 6.8% to 7.4% in semen samples diluted with SM. The viable sperm in DV control after 96 h of storage was 53.3±2.4%; this was higher than SM which was only 23.3±2.4%. The viable sperm in DVT was 59.6±2.3%, significantly higher than DVR, DVF, DV control and SM supplemented with all sugar (Table 2).

Trehalose (disaccharide) and raffinose (trisaccharide) commonly reported to be useful in cryopreservation. In this study shown that trehalose maintained the sperm viability in DV better than other sugar. Viable sperm related with membrane integrity. In most studies revealed that trehalose supplementation in semen extender enhances the membrane integrity of sperm in farm animals (Hu et al., 2009; Khalili et al., 2009; 2010; Tonieto et al., 2010; Jafaroghli et al., 2011). The presence of trehalose in extenders is likely to modulate membrane fluidity by inserting itself into membrane phospholipids bilayer, thus it renders membrane more stable during cooling.

According to our study the sperm motility decreased by 22.2% for every 24 h of observation, regardless the types of sugar or extenders. This was high when compared with only 11%-17.5% in the same species (Masuda et al., 2004). High individual variation of stallion semen was believed responsible for the results of the two studies; different equine breeds had different results. In ram chilled semen the percentage of motile sperm decrease only 12% for 24 h stored (Karahan et al., 2006).
Table 1. Motile sperm of stallion chilled semen (%) in skim milk and Dimitropoulos extenders stored at 5 °C supplemented with different sugar, values represent means (±SD)

| Stored time (Hour) | Skim milk extender | Dimitropoulos extender |
|-------------------|--------------------|------------------------|
|                   | Control | Trehalose | Raffinose | Fructose | Control | Trehalose | Raffinose | Fructose |
| 0                 | 68.2±5.6 | 68.2±5.6 | 68.2±5.6 | 68.2±5.6 | 72.1±3.2 | 72.1±3.2 | 72.1±3.2 | 72.1±3.2 |
| 12                | 48.6±2.3 | 50.5±3.1 | 51.4±2.2 | 56.4±1.9 | 56.7±2.1 | 59.6±2.9 | 58.3±1.8 | 63.8±2.4 |
| 24                | 39.1±1.7 | 41.4±2.1 | 41.4±3.3 | 48.2±1.9 | 48.3±2.2 | 54.2±3.2 | 52.5±1.8 | 58.8±2.2 |
| 36                | 32.3±2.2 | 34.1±1.8 | 37.2±3.2 | 40.5±1.2 | 40.8±2.6 | 45.4±1.1 | 45.8±1.5 | 52.1±2.3 |
| 48                | 25.0±2.1 | 26.4±2.4 | 25.0±3.1 | 31.4±2.2 | 34.6±1.9 | 40.0±1.6 | 39.2±2.1 | 46.3±2.3 |
| 60                | 19.1±3.2 | 19.6±1.7 | 19.6±2.3 | 25.0±1.8 | 27.9±2.4 | 34.2±2.1 | 32.5±2.3 | 39.6±1.5 |
| 72                | 13.0±2.7 | 14.5±1.6 | 13.5±3.2 | 20.0±1.5 | 19.6±2.3 | 26.3±2.2 | 23.6±1.9 | 30.0±2.1 |
| 84                | 9.1±4.1  | 11.4±3.1 | 10.0±2.3 | 16.4±1.9 | 13.3±2.7 | 18.3±2.2 | 16.7±1.9 | 25.0±1.5 |
| 96                | 5.9±3.2  | 6.4±2.1  | 5.0±3.3  | 11.8±2.1 | 6.7±1.3  | 12.2±2.3 | 11.7±2.1 | 17.1±1.8 |

Note: Means in the same row with different superscript differ significantly (P<0.05).

Table 2. Viable sperm of stallion chilled semen (%) in skim milk and Dimitropoulos extenders stored at 5 °C, values represent means (±SD)

| Stored time (Hour) | Skim milk extender | Dimitropoulos extender |
|-------------------|--------------------|------------------------|
|                   | Control | Trehalose | Raffinose | Fructose | Control | Trehalose | Raffinose | Fructose |
| 0                 | 78.8±3.0 | 78.8±3.0 | 78.8±3.0 | 78.8±3.0 | 79.9±4.0 | 79.9±4.0 | 79.9±4.0 | 79.9±4.0 |
| 12                | 59.9±1.7 | 64.8±0.9 | 59.5±3.1 | 66.5±2.7 | 72.6±3.0 | 73.8±1.7 | 74.4±2.4 | 73.5±3.0 |
| 24                | 51.7±2.3 | 54.6±1.5 | 51.6±1.8 | 54.9±2.6 | 70.1±2.4 | 69.0±1.9 | 69.3±2.9 | 70.7±1.7 |
| 36                | 44.8±2.8 | 47.9±3.8 | 45.6±2.9 | 49.3±2.2 | 66.9±1.5 | 67.2±2.1 | 65.8±1.3 | 68.2±2.3 |
| 48                | 40.4±2.4 | 42.4±2.8 | 39.7±1.5 | 43.3±2.3 | 63.5±3.0 | 64.8±2.1 | 63.8±2.0 | 64.3±1.6 |
| 60                | 36.7±2.4 | 37.5±1.2 | 34.5±1.3 | 36.8±1.6 | 61.4±0.4 | 62.4±3.4 | 61.3±9.2 | 62.2±1.9 |
| 72                | 33.7±3.4 | 31.3±4.3 | 31.0±2.6 | 33.0±2.8 | 57.5±3.1 | 60.8±2.2 | 58.3±3.1 | 58.8±1.4 |
| 84                | 29.3±2.8 | 24.3±2.4 | 26.2±3.1 | 27.4±2.6 | 54.8±2.2 | 59.4±2.4 | 58.1±2.5 | 57.3±2.1 |
| 96                | 23.3±2.4 | 16.3±3.4 | 20.9±2.1 | 20.8±3.1 | 53.3±2.4 | 59.6±2.3 | 58.5±3.1 | 56.9±2.5 |

Note: Means in the same row with different superscript differ significantly (P<0.05).

The Sperm Survival of Stallion Chilled Semen Stored at Ambient Temperature

At ambient temperature percentage of motile sperm decreased by 8.4% to 10.8% for every three hours stored. The highest decrease was 15.0% to 30.5%; this occurred during the first three hours. At the 18th h of observation, the percentage of motile sperm in DVF was 21.4±2.1% higher than DVR (15.1±3.2%), DVT (14.5±2.2%) and DV (11.9±2.3%), SMF (8.6±2.1%), SMR (4.1±1.8%) and SMT (5.5±1.9%) (Table 3).

Viable sperm decreased by 7.0% to 9.1%, the decrease was smaller compared to motile sperm (Table 4). At the 18th h of stored, the viable sperm in DVF was 37.7±1.5%; this was higher than DV (30.6±1.8%), DVT (28.6±2.7%) and DVR (28.6±2.7%). No significant difference between DVF, DVT and DVR. Supplementation of fructose and raffinose in SM were (29.5±3.4%) and (27.0±3.1%), both were significantly higher than trehalose (26.1±3.1%), or SM (24.1±1.2%).

In our laboratory the ambient temperature is between 24 °C until 29 °C. Sperm survival is very low; this is due to sperm reach nearly optimum cellular activity, consuming the energy substrates, leads to lactic acid accumulation as metabolic waste. According to Vishwanath & Shannon (1999), the main source of peroxidation occurring at ambient temperature in bovine sperm is oxidative deamination of aromatic amino acid by aromatic amino acid amine (AAA0), which is released from dead sperm plasma membrane. The enzyme is increased with increasing temperature and duration of storage. The amount of AAA0 enzyme is increased along with the temperature increase and prolonged duration of storage, AAA0 enzyme is inactive in viable sperm. This mechanism might be happen the same way in equine sperm. The explanation of low sperm survival at ambient temperature (only 18 h); this is related to the lack of energy substrates, lactic acid accumulation, and AAA0 release by dead sperm plasma membrane.

Semen extender is a media for sperm during storage; the semen extender components directly influence the survival of sperm. Dilution with DV demonstrated better results than SM in semen samples; these were caused by the two types of sugar (fructose and glucose).
present in DV extender, whereas only glucose present in SM extender. DV extender also contains egg yolk, which has lecithin and phospholipids to protect sperm during cold shock (Aboagla & Terada, 2004b). Extenders containing egg yolk superior compared to extenders without egg yolk on the sperm movement after 48 h of storage (Katila et al., 2004). Other than sugars and egg yolk, DV extender contains citrate buffer to neutralize sperm metabolism waste (Katila et al., 2004).

The advantages of DV extender in this study were agreement with the ones reported by Yudi et al. (2008) and Parlevliet et al. (1992); this group of researchers demonstrated that DV extender showed progressive sperm percentage in Dutch warm blood stallion semen stored at 5 °C and 20 °C better when compared to SM extender.

Milk contains natural buffer (McKinnon, 1999), which may have less effects than citrate buffer. Sperm break down glucose, fructose, or mannose to lactic acid under anaerobic (absence of oxygen) condition. Glycolysis (fructolysis) allows sperm to survive during storage prior to insemination; this makes fructose (a simple sugar) in seminal plasma is the most important component to be metabolized by sperm. In this study, fructose had better effects than glucose (control) and raffinose or trehalose, in all extender stored at 5 °C and 20 °C better when compared to SM extender.

| Stored time (Hour) | Control | skim milk extender | Dimitropoulos extender |
|-------------------|---------|--------------------|------------------------|
|                   | Trehalose | Raffinose | Fructose | Control | Trehalose | Raffinose | Fructose |
| 0                 | 68.2±2.2 | 68.2±2.2 | 68.2±2.2 | 71.7±2.0 | 71.7±2.0 | 71.7±2.0 |
| 3                 | 40.0±1.9  | 41.4±2.3  | 37.7±3.1  | 50.4±2.1  | 51.3±2.1  | 52.5±1.4  |
| 6                 | 29.6±3.1  | 32.3±1.7  | 26.8±2.1  | 38.4±1.9  | 39.4±2.1  | 39.1±2.2  |
| 9                 | 22.3±2.3  | 25.0±3.0  | 20.5±2.4  | 30.5±2.3  | 30.8±1.8  | 34.1±1.8  |
| 12                | 14.6±1.9  | 16.4±2.3  | 14.1±2.1  | 25.1±3.2  | 27.5±2.1  | 27.6±1.4  |
| 15                | 9.1±3.1   | 11.4±2.9  | 9.1±3.1   | 18.3±2.0  | 21.2±2.0  | 20.8±1.8  |
| 18                | 3.6±2.5   | 5.5±1.9   | 4.1±1.8   | 11.9±2.3  | 14.5±2.4  | 15.1±3.2  |

Note: Means in the same row with different superscript differ significantly (P<0.05).

| Stored time (Hour) | Control | Skim milk extender | Dimitropoulos extender |
|-------------------|---------|--------------------|------------------------|
|                   | Trehalose | Raffinose | Fructose | Control | Trehalose | Raffinose | Fructose |
| 0                 | 78.8±5.7 | 78.8±5.7 | 78.8±5.7 | 79.9±4.0 | 79.9±4.0 | 79.9±4.0 |
| 3                 | 65.2±3.2  | 66.5±1.7  | 65.3±3.7  | 70.8±2.8  | 69.9±1.4  | 67.8±1.1  |
| 6                 | 56.3±3.2  | 59.5±1.6  | 58.4±2.6  | 61.9±1.7  | 61.4±2.2  | 59.8±1.2  |
| 9                 | 44.9±2.0  | 50.0±2.0  | 47.1±1.3  | 53.7±1.8  | 53.9±1.9  | 51.9±2.3  |
| 12                | 38.0±2.5  | 40.6±2.4  | 40.5±2.6  | 46.2±2.4  | 44.8±1.7  | 42.2±1.6  |
| 15                | 30.5±1.4  | 32.9±2.2  | 34.0±3.1  | 38.8±3.3  | 37.3±1.0  | 36.8±1.8  |
| 18                | 24.1±1.2  | 26.1±2.1  | 27.0±3.1  | 30.6±1.8  | 28.6±2.7  | 28.4±1.2  |

Note: Means in the same row with different superscript differ significantly (P<0.05).
Stallion sperm are more fragile to cold shock than sperm of other species; this is caused by high stallion plasma membrane concentration of arachidonic fatty acid (Chow et al., 1986). Bull, ram, and stallion sperm plasma membrane arachidonic fatty acid concentrations are 3.5%, 4.5%-5% (White, 1993), and 18.2% (Chow et al., 1986). Arachidonic acid is an unsaturated fatty acid with a 20-carbon chain and four double bonds. High fatty acid concentration in stallion sperm causes the instability of plasma membrane making it is easily damaged by reactive oxygen species (ROS) leading to lipid peroxidation (Neild et al., 2005; Ball, 2008). Lipid peroxidation increases membrane plasma rigidity, decreases enzyme binding affinity (includes disturbing ion pump), disturbs membrane receptor activity and permeability (Best, 2006).

Centrifugation to separate seminal plasma in the early processing is believed to be one of the factors contributing to the low stallion sperm survival (Kopt et al., 2005; Morrell et al., 2009). Three enzymes present in stallion seminal plasma are superoxide dismutase (SOD), catalase, and glutation (Baumber et al., 2003); these enzymes may reduce lipid peroxidation induced by ROS, and preserve sperm metabolism. For instance, SOD combined with peroxidase convert superoxide anion to oxygen, water, and hydrogen (Best, 2006).

Both ROS and cold shock contributed to the sperm damage during storage at 5 °C. Compared to the storage at ambient temperature, a 4-5 °C decrease in temperature interrupts the sperm metabolism, prolonging survival of sperm. Cold shock alters normal configuration of sperm membrane to hexagonal configuration (Ball, 2008) this damages sperm plasma membrane. When the damage occurs in midpiece, aspartate aminotransferase (AspAT), which is the main enzyme in ATP production by mitochondria, is released to the seminal plasma. Loss of AspAT interrupts ATP production and disturbs sperm motility (Colenbrander et al., 1992).

CONCLUSION

The mitomopulos (DV) extender better preserved the quality of stallion chilled semen at both ambient temperature and 5 °C. Fructose supplementation improved the sperm motility of stallion chilled semen stored at both ambient temperature and 5 °C and trehalose protect viable sperm only in DV at 5 °C.

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