Kinematics motility of friesian-holstein sperm sexing in l-ascorbic acid treatments

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Abstract. The study aimed to examine the kinematics motility and recovery rates of frozen-thawed Friesian Holstein bull sexing semen in the addition of l-ascorbic acid treatment. Semen collected from Friesian Holstein bulls with the minimum standard of sperm motilities for sexing sperm (60%), and abnormality (<20%) used for this study. Semen was diluted with final concentration 300x10^6 sperm/ml in Brackett-Oliphant (BO) medium. The sperms were sorted using the BSA column method. Sexing sperm were extended with Tris egg yolk (TEY) extender in addition L-Ascorbic Acid (LA) 0%, 0.25%, 0.5%, and 0.75% before freezing process. Frozen sexing semen was thawed for the kinematics motility evaluation with parameter; Tmot, Pmot, velocity and distance compared to control group, 0.25% and 0.5% TEY-LA. The recovery rate 0.5% TEY-LA had highest values between among all treatment in X and Y frozen sexing semen. In conclusion, the addition of 0.5% l-ascorbic acid to tris egg yolk extender could be advantageous tool to improve the kinematics motility and increased recovery rates of Friesian Holstein sexing sperm.

1. Introduction
The pre-sex selection of offspring in farm animals is of high commercial value. The pre-selection method of sex in animals could be done on the sorting of sperm bearing X and Y before insemination. Frozen semen for artificial insemination (AI) program has the advantage that it can be used for a long time, but the quality of the semen after freezing can decrease by several factors, one of the factors was caused by frozen sexing semen process [1]. Frozen semen sexing production for artificial insemination program consisted of a series of the process including separating sperm bearing x and y, semen dilution in freezing extender, equilibration and freezing process and post-thawing evaluation [2]. During cooling and freezing process, and sperm cells are susceptible to get the harmful effect from unbalanced osmotic pressure, protein denaturation, cellular acidosis, energy loss, membrane breakdown, crystallization of cells body, destabilization of the cytoskeleton and formation of free radicals or reactive oxygen species (ROS) [1].

Antioxidants are molecules that inhibit the formation of ROS and lipid peroxidation. Superoxide dismutase, glutathione peroxidase, and catalase are well-known antioxidants significant for sperm function because they protect sperm cells from oxidative stress [3]. The supplementation of cryopreservation extenders with antioxidants provides a cryoprotective effect on the bull, ram, goat,
boar, canine, and human sperm quality, thus minimizing ROS's detrimental effect, and improving quality of post-thaw sperm [4]. One of the antioxidants that can improve semen quality is L-ascorbic acid (LA), LA can strengthen tissue stability plasma membrane against its peroxidation during frozen semen processing because there is direct contact with oxygen (O$_2$), which can cause death in sperm [5]. L-ascorbic acid acts as an antioxidant to prevent oxidation reactions from other molecules and neutralizes the effects of H$_2$O$_2$ on DNA, recycles inactive vitamin E, and reduces lipid peroxidation [6]. L-ascorbic acid was water soluble vitamin which was able to protect sperm from damage caused by oxidative stress by neutralizing hydroxyl, super-oxidation and peroxide hydrogen radical and prevent sperm agglutination [7].

The addition of LA to semen extender improved sperm motility and viability after cryopreservation of bull semen [8], and combinations of LA with either catalase or reduced glutathione to Tris extender improve the post-cryopreservation semen quality of Holstein bulls [9] improve quality X and Y sperm of dairy cattle (Bos taurus) after thawing [10]. The main purpose of this research was to examine the post-thawing kinematics motility and recovery rates of frozen-thawed Friesian Holstein bull sexing sperm in the addition of LA treatment.

2. Methods

2.1 Semen Collection

Semen was collected from Friesian Holstein Bulls from Research Center for Biotechnology LIPI by using artificial vagina then evaluated the quality; volume, concentration, motility, and sperm abnormality using objective microscopy prior to freezing. The sperm motilities for sexing sperm minimum 60% and abnormality sperm <20% [2] were used for this study.

2.2 Sex sperm separation BSA Column

Semen concentration was diluted with final concentration 300x10$^6$ sperm/mL in Brackett-Oliphant (BO) medium then sperm were separation by using modification BSA (Bovine serum albumin) column method with proportion 5 % (Top fraction): 10 % (Bottom fraction) then semen was incubated for 60 minutes at water-bath 37°C [2,10]. Thereafter, each sperm fraction was collected then centrifuged at 1800 rpm for 10 minutes. Sexing sperm was diluted with Tris egg yolk extender then added L-Ascorbic Acid with concentration 0% (control), 0.25%, 0.5% dan 0.75%. Sexing sperm then was filled in mini straw 0.25 mL then did an equilibration at 4°C for 2 to 4 hours, pre-freezing for 15 minutes and kept in liquid nitrogen at -196°C.

2.3 Sexing sperm kinematics evaluate by using Computer Assisted Sperm Analyzer (CASA)

Frozen sexing semen were thawed in water-bath at 37°C for 30 seconds after 24 hours stored in liquid nitrogen -196°C. Sperm were then evaluated using Computer Assisted Semen Analyzer (CASA: Spermvision™ 3.7.8 Minitube Germany) set at magnification of 200 X with the following parameter kinematics motility: the percentage of motile sperm (Tmot %); the percentage of progressive motility sperm (Pmot %); Velocity Average Path (VAP: µm/s); Velocity Curvilinear (VCL: µm/s); Velocity Straight Line (VSL: µm/s); Distance Average Path (DAP: µm/s); Distance Curvilinear (DCL: µm/s); Distance Straight Line (DSL: µm/s); Linearity (LIN), Amplitude of the Lateral Head (ALH) and Beat Cross Frequency (BCF) [11–13].

2.4 Recovery rate sperm motility

The recovery rate value was obtained from the motility values of post thawing sperm divided by the motility value of fresh sperm at 100% [14].

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\text{Recovery rate} = \frac{\text{Post thawing motility} \times 100\%}{\text{Fresh semen motility}} \tag{1}
\]
2.5 Statistical analysis
Sperm motility data were presented as means ± standard deviation (SD) and recovery rate value were analyzed descriptive statistic and for kinematics motility data analyzed by one way analysis of variance (ANOVA) using Minitab 17 for Windows. Differences between treatments were considered statistically significant at P<0.05 by Fisher LSD analysis.

3. Results and Discussions
Motility evaluation in fresh semen is a standard assessment to measure whether fresh semen can be processed for frozen semen and artificial insemination programs. The total motility of fresh sperm, after separation of X and Y and frozen sexing semen (table 1). This study showed the motility of fresh semen has a value above the minimum standard of fresh semen motility used for frozen semen production. According to the Indonesian national standard (SNI 4869-1:2017), the minimum motility of fresh semen used for frozen semen production is 70%, and frozen semen motility for artificial insemination programs 40% [14].

Table 1. Sperm motility of fresh semen, after sex sorting and frozen thawed.

| Parameter              | Motility (Mean ± SD) |
|------------------------|-----------------------|
| Fresh Semen (%)        | 81.26 ± 2.3           |
| After sex sorting (%)  |                       |
| X Sperm                | 74.42 ± 3.44          |
| Y Sperm                | 66.15 ± 7.41          |
| Frozen-thawed (%)      |                       |
| X Sperm                | 49.34 ± 8.87          |
| Y Sperm                | 45.43 ± 10.06         |

Table 2. Kinematics motility of post thawed X sexing sperm.

| Parameter | Treatment (Mean ± SD) |
|-----------|-----------------------|
|           | Control               | Tris Egg Yolk L-Ascorbic Acid (TEY-LA) |
|           |                        | 0.25% | 0.50% | 0.75% |
| Tmot (%)  | 48.87±6.22ab          | 52.25±8.22ab | 58.73±2.18a | 37.53±16.4b |
| Pmot (%)  | 46.39±6.44ab          | 50.02±8.57ab | 56.57±2.81a | 35.35±15.53b |
| VAP (µm/s)| 47.56±7.08ab          | 53.71±12.38a | 48.9±5.45ab | 36.48±1.3b |
| VCL µm/s  | 76.77±10.57ab         | 89.34±20.13a | 79.37±11.46ab | 54.41±6.16b |
| VSL µm/s  | 36.55±4.97ab          | 41.78±9.98a | 38.63±6.06ab | 28.51±1.31b |
| LIN (%)   | 48.3±6.51a            | 46.3±0.58a | 48.3±2.89a | 52.7±8.96a |
| ALH       | 4.29±0.95a            | 4.6±0.88a  | 3.96±0.26a | 4.03±0.99a |
| BCF (Hz)  | 17.6±3.75ab           | 18.87±3.90a | 18.79±2.09a | 11.64±3.34b |
| DAP (µm/s)| 20.45±4.21ab          | 23.16±5.47a | 21.4±3.19a | 15.11±1.28b |
| DCL(µm/s) | 33.24±10.72ab         | 38.68±8.87a | 35.03±6.39ab | 22.81±3.78b |
| DSL (µm/s)| 15.68±3.09ab          | 18.04±4.50a | 16.92±3.31ab | 11.75±6.1b |

a,bMean±SD with different superscript within row is differ significantly (P<0.05).

In the present study showed a decrease gradually in fresh sperm motility from 81.26 ± 2.3% to 66.15 ± 7.4174.42 ± 3.44% after the x and y separation process then to 45.43 ± 10.06-49.34 ± 8.87% after the freezing process (table 1). This study indicates that decreased motility occurred during the process of
separating sperm into frozen semen was a normal process and decreasing not more than 50%. The decrease in quality is very high, about 50% of sperm will die during freezing, and sperm that survive generally have low fertility [15].

The total motility (Tmot) and progressive motility (Pmot) 0.75% TEY-LA treatment had the lowest values between among all treatment values (P<0.05) in X and Y sexing sperm (Tables 2 and 3), while sexing sperm in 0.25%, 0.75% TEY-LA and control group were not significantly different (tables 2 and 3). The Tmot values 0.75% and 0.25% TEY-LA were not qualified for minimum post thawing motility values according to SNI 4869-1:2017 with 40%. Motility is a standard assessment to measure whether frozen semen can be used for artificial insemination programs with 40% minimum PTM and 32% progressive motility [14,16].

In the present study Tmot and Pmot value 0.5% TEY-LA treatment had the highest values (P<0.05) compared to 0.75% TEY-LA (table 2) and between among all treatment values (P<0.05) (Table 3), which confirmed that 0.5% LA was sufficient improved sperm quality such a result consistent with the previous report [8,10]. Reduction in sperm motility is mainly due to mitochondrial impairment and generation of reactive oxygen species (ROS) [17].

The sperm sexing kinematic motility namely, VAP, VCL, VSL and BCF were affected by the addition of LA (P>0.05). The VAP, VCL and VSL values were the highest with 0.25% TEY-LA in X sexing sperm (VAP: 53.71±12.38, VCL: 89.34±20.13, VSL: 41.78±9.98) and control group in Y sperm sexing (VAP: 62.86±5.72, VCL: 114.25±18.12, VSL: 47.73±1.93) respectively as presented in Tables 2 and 3. The VAP values in this present study showed 0.75% TEY-LA had the lower values between among all treatments (P<0.05) and the VCL values in this study showed 0.25% TEY-LA X sexing sperm and control group Y sexing sperm (tables 2 and 3) were highest values (P<0.05).

### Table 3. Kinematics motility of post thawed Y sexing sperm.

| Parameter | Control | 0.25% | 0.50% | 0.75% |
|-----------|---------|-------|-------|-------|
| Tmot (%)  | 43.29±4.99b | 39.98±4.14b | 60.2±10.97a | 38.25±4.28b |
| Pmot (%)  | 41.74±5.08b | 37.86±4.16b | 58.49±9.98a | 35.63±3.41b |
| VAP (µm/s)| 62.86±5.72a | 43.34±4.26bc | 51.06±6.08b | 38.04±2.22c |
| VCL µm/s | 114.25±18.12a | 72.4±4.98b | 81.8±15.21b | 63.82±7.74b |
| VSL µm/s | 47.73±1.93a | 34.36±4.78c | 40.81±3.94b | 28.14±1.57c |
| LIN (%)  | 42±6.56a | 46.3±5.13a | 50±5.29a | 44±4.36a |
| ALH      | 4.31±1.05a | 3.29±0.8a | 3.64±0.96a | 3.14±0.42a |
| BCF (Hz) | 23.94±1.15a | 17.8±2.94b | 19.1±2.08b | 16±1.36b |
| DAP (µm/s)| 28.26±1.75a | 19.5±3.03bc | 22.65±2.2b | 17.04±0.93c |
| DCL(µm/s)| 51.54±6.89a | 32.75±4.44b | 36.43±6.37b | 28.64±3.38b |
| DSL (µm/s)| 21.5±0.63a | 15.42±2.96bc | 18.14±1.39b | 12.58±0.68c |

*Mean±SD with different superscript within row is differ significantly (P<0.05).

Different results occur in VSL values (tables 2 and 3) the control group and LA treatment were above minimum VSL value 25 µm/s. The minimum standard velocity values for assessing fertility potential were VCL> 70 µm/s, VAP> 45 µm/s, VSL> 25 µm/s [16]. Kathiravan et al. [18] reported that only the parameters VCL, VSL, VAP could be used to predict in vivo fertility. Another report presumed that VCL is related to the ability of sperm to penetrate cervical mucus [19]. Moreover, post-thawed sperm VSL is related directly to bull fertility [20].
The Linearity (LIN) and Amplitude of the Lateral Head (ALH) in this study were not affected significantly (Tables 2 and 3). The ALH value in this study were less than 7 μm, which means that sperm were not showed hyperactivity that reduced sperm progressive movement ability. Kathiravan et al. [18] reported greater values of VCL with ALH is not desired ALH ≥ 7 μm because it was indicated by hyper-activated sperms.

The BCF was the frequency of movement the head of sperm from side to side during the measurement period in hertz unit. The BCF value in this study showed the 0.75% TEY-LA was lower than 0.25% 0.50% TEY-LA and control group (table 2), otherwise control group significantly different (P<0.05) compared TEY-LA treatment. The BCF is an indicator of the strength of sperm the addition of l-ascorbic acid was decreased the head sperm movement frequency. More recent report suggested that sperm motion characteristics, such as total motility, progressive motility or BCF, were found to be predictors of bovine in vivo fertility [21].

The distance value in this study were affected by the addition of l-ascorbic acid the DAP value ranging (15.11 μm/s - 23.16 μm/s), DCL (22.81 μm/s – 38.68 μm/s) and DSL (11.75 μm/s - 18.14 μm/s) respectively (Tables 2 and 3). The distance values in this study were had linear trend with velocity values and can indicated sperm velocity.

![Figure 1](image-url)  
**Figure 1.** Recovery rate sexing sperm after l-ascorbic acid addition

Recovery rate (RR) was the ability of sperm to recover from freezing and used to indicate the successful cryopreservation. The highest RR value was 0.5% TEY-LA treatment in X and Y sexing sperm compared to control, 0.25% and 0.75% TEY-LA (figure 1). The addition 0.25% TEY-LA in X sexing sperm and 0.75% TEY-LA in XY were not qualified minimum recovery rates value. In the present study showed the addition 0.5% l-ascobic acid in frozen semen extender can increased sperm ability to recover from freezing. Recovery rate was the number of sperm from frozen semen that have recovered after thawing with a minimum recovery rate of 50% [14].

4. Conclusion  
In conclusion, the addition of 0.5% l-ascobic acid to tris egg yolk extender could be advantageous tool to improve the kinematics motility and increased recovery rates of Friesian Holstein sexing sperm.

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