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Motility characterization of albumin sexed spermatozoa in two different diluents and additional antioxidant

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ABSTRAK

Tujuan penelitian ini adalah mengkarakterisasi pola gerakan spermatozoa hasil sexing (X dan Y) menggunakan albumin dengan perlakuan pengencer dan antioksidan. Materi yang digunakan dalam penelitian ini adalah semen segar sapi Peranakan Ongole (PO) dengan motilitas progresif >70%. Metodologi sexing yang digunakan adalah gradien albumin (putih telur) 5%, 10% dan 15%. Pengencer yang digunakan adalah CEP-2 dan andromed; dengan atau tanpa penambahan antioksidan Glutation 1mM. Semen hasil sexing dibuat semen cair dan disimpan pada suhu 3-5°C. Motilitas spermatozoa semen cair hasil sexing diamati pada hari ke-0 (H0) dan ke-5 (H5). Analisis motilitas spermatozoa menggunakan SCA v.2.1. Parameter yang diukur meliputi: motilitas total , motilitas progresif, Velocity Curve Linear (VCL), Velocity Straight Linear (VSL), Velocity Average Pathway (VAP), Linearity (LIN), Straightness (STR), Wobble (WOB), Amplitude Lateral Head (ALH), Beat Cross Frequency (BCF) dan hiperaktif spermatozoa. Rancangan penelitian menggunakan pola factorial 2 x 2, sebagai faktor pertama adalah jenis pengencer (CEP-2 dan Andromed) dan faktor kedua adalah penggunaan antioxidant Glutathione (dengan atau tanpa). Analisis data menggunakan General Linear Model (GLM), SPSS-IBM 24. Semen hasil sexing dengan pengencer CEP-2 menunjukkan motilitas spermatozoa yang lebih baik daripada andromed pada lapisan atas (spermatozoa X) dan lapisan bawah (spermatozoa Y). Pengencer CEP-2 mempunyai peranan besar dalam menjaga motilitas, motilitas progresif dan kecepatan spermatozoa selama penyimpanan dingin. Glutathione 1 mM dapat mendukung motilitas spermatozoa sexing pada penyimpanan dingin, khususnya nilai LIN, STR dan WOB.

Kata kunci : moilitas, spermatozoa, sexing, albumin

ABSTRACT

The purpose of this study was to characterize spermatozoa motility of sexed semen (X and Y) using albumin by diluents and antioxidant treatments. The material used in this study was fresh semen of Ongole Crossbreed (OC) bull with progressive motility ≥70%. The sperm sexing methodology used albumin gradient of 5%, 10%, and 15%. The diluents used were CEP-2 and andromed with or without the addition of 1mM Glutathione as an antioxidant. The sexed semen was made into liquid semen and stored at 3-5°C. The motility was observed at day 0 (H0) and day 5 (H5). Motility was analyzed using SCA v.2.1. The parameters measured were total motility, progressive motility, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF and sperm hyperactive. The experimental applied was the factorial pattern 2 x 2. The first factor was the type of diluents (CEP-2 and Andromed), and the second factor was additional glutathione (with or without). Data was analyzed by general linear model of SPSS-IBM 24 program. Sexed semen with CEP-2 diluents showed better motility of spermatozoa than andromed in the upper layers (spermatozoa X) and lower layers (spermatozoa Y). The CEP-2 diluent had a big role to maintain

motility, progressive motility and velocity spermatozoa during cold storage. The addition of Glutathione 1mM could support the motility of sexed spermatozoa at cold storage, especially for LIN, STR, and WOB values .

Keywords: motility, sperm, sexing, albumin

INTRODUCTION

Bulls are commodities that are expected to answer the level of consumer demand for meat. On the other hand, the role of the cow is very large in producing calves for breeding. Reproductive biotechnology that can support an increase in bulls and cows populations is sperm sexing technology. Through sperm sexing technology, stakeholders can manage the calf sex that they want to produce.

Sperm sexing using albumin is a relatively easy sperm sexing method. There are many principles works base of sperm sexing, such as: size, motility, surface charge and chromosome fluorescence, electricity, weight density, surface of macromolecular proteins, effects of atmospheric pressure, DNA content and different pH effects (Ondho and Udrayana, 2018: Bhalakiya *et al.,* 2018; Ogbeuwu *et al.,* 2010). The principle works based on the ability movement of spermatozoa (motility). Albumin gradient is an effective method that can increase proportion of sperm motility and relieve abnormal sperm (Bhalakiya *et al.,* 2018). Based on the capability of motile spermatozoa, it is known that spermatozoa X (females) have large head sizes so that their motility slower and less capable to penetrate albumin (upper layer). While spermatozoa Y with a small head size allows more movement to be able to penetrate albumin (lower layer). Nevertheless, the study of Carvalho *et al.* (2009) and Penfold *et al.* (1998) stated that there were no differences between spermatozoa X and Y, except for swimming patterns (linearity and straightness). Many methods can be used for sperm sexing, which were gradient percoll, gradient albumin, flow cytometry, electrophoresis etc (Susilawati, 2014). Previous sperm sexing studies have been conducted using stratified albumin concentrations including: 30% and 10% (Pratiwi *et al.,* 2007); 50%, 30% and 10% (Susilawati, 2014). Hafez and Hafez (2000) stated that sperm sexing with albumin produced spermatozoa Y as much as 75-80%.

Caudal Epididymal Plasm (CEP) is a diluents composed of ion that resembles the seminal fluid in the cauda epididymis. Ratnawati (2017) stated that CEP-2 can support the motility of spermatozoa rather than tris aminomethane and skim milk in cold storage. Andromed is a commercial diluent which is practical and efficient to be used. Purwoistri *et al.* (2013) stated that andromed can produce the same abnormalities, viability, concentration, and total motile spermatozoa as CEP-2 diluents plus 10% egg yolk. Andromed and CEP-2 with 10% egg yolk can increase the quality of spermatozoa.

Components in seminal fluid that can support the life of spermatozoa, including anti-oxidants. Endogenous antioxidant components (enzymatic and non-enzymatic antioxidants) are not enough to help maintain semen quality during storage, so exogenous antioxidants are needed. One of the antioxidants that can be used is glutathione. Glutathione is a non-enzymatic antioxidant that protects spermatozoa from reactive oxygen species (ROS) during storage. Ansari *et al.* (2014), El-kon and Darwish (2011), and Tuncer *et al.* (2010) stated that the dose of glutathione that can be used during storage in cattle and buffaloes are 0.5 or 1 mM.

The purpose of this study was to characterize spermatozoa motility of sexed semen (X and Y) using albumin with diluents and antioxidant treatments.

MATERIALS AND METHODS

This research was approved by Balitbangtan/Lolitsapi/Rm/01/2018. The material research used 5 Ongole Crossbreed (OC) bulls with 10 replications. The research location is the Reproductive Laboratory of Beef Cattle Research Station started in July-October 2018. Collecting semen was done by using an artificial vagina and continue with a fresh semen analysis. The CEP-2 was prepared in the Reproduction Laboratorium of Beef Cattle Research Institute. The ingredients of CEP-2 consisted of: NaCl 15 mmol/L, KCl 7 mmol/L, $CaCl_2(H_2O)_2$ 3 mmol/L, $MgCl_2(H2O)_6$ 4 mmol/L, $NaHCO_3$ 11.9 mmol/L, NaH_2PO_4 8 mmol/L, KH_2PO_4 20 mmol/L, Fruktosa 55 mmol/L, Sorbitol 1 g/L, Tris 133,7 mmol/L,

gentamicin-S 0,05 g/L, asam sitrat 42 mmol/L, kuning telur 10% dan putih telur 0,4% (Ratnawati, 2017). Andromed was prepared by mixing it with aquades in a ratio of 1: 4. The requirement for fresh semen to be processed into liquid semen is progressive motility of spermatozoa ≥70%.

The sperm sexing technique used gradient albumin 5%, 10% and 15%. The treatment was two types of diluent (CEP-2 and andromed) and addition 1 mM glutathione antioxidants (with-without). The stages of sperm sexing are listed in Figure 1. Movement patterns of spermatozoa were assessed objectively using Sperm Class Analyzer (SCA v. 2.1) during cold storage at day 0 and 5. A liquot 3-4 µl of semen was deposited on a warmed slide at 38°C and covered with a coverslip. The microscope was set at phase contrast at pH 1 and magnification 10 x 10. The reflector mirror was coated with a green filter. The diaphragm and light intensity were set at the color standards specified by the microscope. Sperm images in 5 fields were digitized for analysis the sperm kinematic pattern (Ratnawati *et al.*, 2018).

The parameters measured in this study were spermatozoa motility (motility, progressive motility, VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF, sperm hyperactive). The experimental design used factorial pattern 2 x 2. The first factor was the type of diluents (CEP-2 and Andromed). The second factor was additional glutathione (with or without). Data were analyzed by general linear model of the SPSS-IBM 24 program.

RESULTS AND DISCUSSIONS

During cold storage, sexed spermatozoa motility was daily observed and analyzed by using CASA until day 5 (H5).

Total Motility and Progressive Motility

During cold storage, observations of spermatozoa motility in the upper (X) and lower (Y) layers are listed in Table 1 and Table 2. It is known that there was no interaction between the type of diluents and the use of antioxidants (glutathione) to the progressive motility and total spermatozoa motility in the upper and lower layer. There was a significant difference in the value of motility and progressive motility spermatozoa at days 0 and 5 between with CEP-2 diluents and andromed in the upper and lower layer. The value of motility and progressive motility with CEP-2 diluents was higher than andromed. The decrease of motility and progressive motility of spermatozoa with andromed diluents were faster

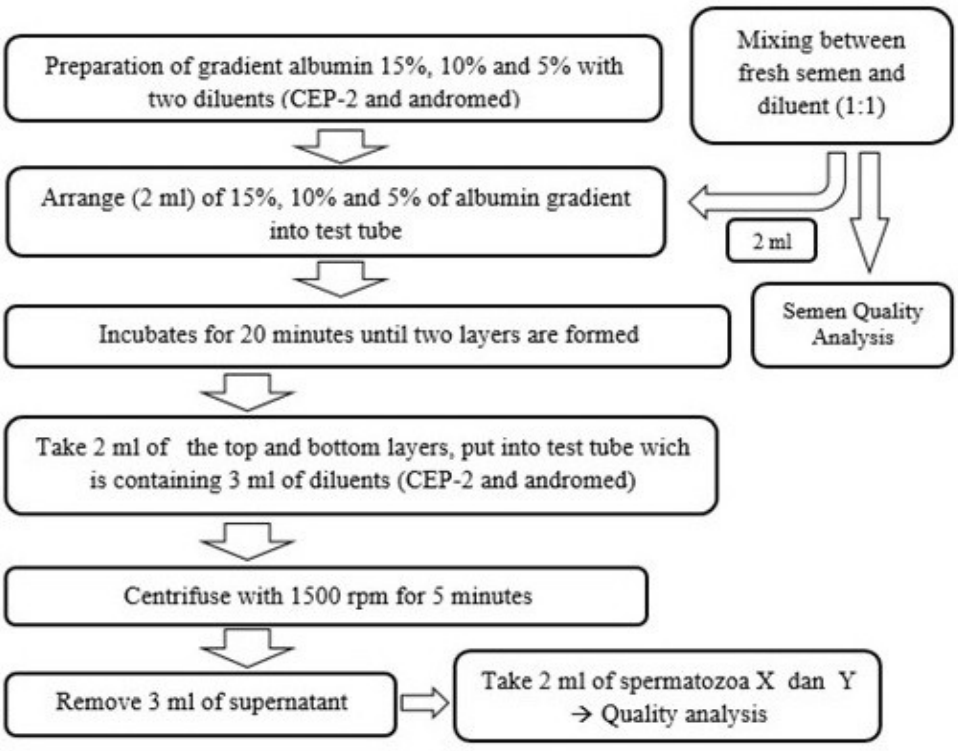

Figure 1. Schematic of Spermatozoa Sexing

Table 1. Total Motility and Progressive Motility of Spermatozoa in the Upper Layer (X)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
Motility (%)	0	97.6	96.7	97.6	96.7	ns	ns	ns
	5	77.3	63.9	74.8	66.5	*	ns	ns
Progressive Motility (%)	0	80.7	69.1	75.4	74.4	*	ns	ns
	5	38.3	24.2	33.6	28.9	*	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; *= significant (P<0.05); ns= non significant (P>0.05)

Table 2. Total Motility and Progressive Motility of Spermatozoa in the Lower Layer (Y)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
Motility (%)	0	99.2	95.8	97.1	97.9	*	ns	ns
	5	85.3	60.4	74.8	70.9	*	ns	ns
Progressive Motility(%)	0	77.1	67.8	72.9	71.9	*	ns	ns
	5	41.0	21.0	31.5	30.5	*	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; *= significant (P<0.05); ns= non significant (P>0.05)

than CEP-2. Whereas, decrease of spermatozoa motility and progressive motility with CEP-2 diluents were lower and could survive until day 5 of cold storage. Meanwhile, the addition of the glutathione did not affect the motility and progressive motility of spermatozoa.

Seidel (2012) stated that sperm progressive motility is the most appropriate motility to be evaluated. During cold storage, spermatozoa metabolism keeps running by producing ROS, which affects to the spermatozoa membrane integrity. The spermatozoa membrane integrity affects the motility of spermatozoa (Ratnawati, 2017). It was contrary to Morrell et al. (2018) study, stated lack of ROS indicated low sperm metabolism activity and result in low pregnancy. The length of cold storage, can decreases spermatozoa motility in the upper and lower layers of sexed semen. At the beginning of storage, energy sources were available and lead to a decrease during storage. This is supported by the composition of CEP-2 diluent ingredients which was conducive to spermatozoa life during cold storage (Ratnawati, 2017). Andromed was easy to be prepared, nevertheless andromed can not maintain and support the motility and progressive motility of sexing spermatozoa during cold storage. The glycerol composition in andromed tend to be toxic to spermatozoa in cold storage.

Kusumawati *et al.* (2017) stated that decreasing motility occurred because of various treatments, such as: the process of separation, washing and cooling that causes sperm to require a lot of energy to maintain physiological conditions. Rubessa *et al*. (2016) stated that during freezing or thawing processes, spermatozoa was injured leading to a reduction of its motility. The damaged of the spermatozoa membrane caused a negative effect on the function of mitochondria to produce ATP. So that, this damaged interfere with the metabolic processes that will affect the decrease of the spermatozoa movement (Susilawati, 2014; Tirpak

et al. 2015).

Velocity (VCL, VSL, VAP)

Observation of velocity spermatozoa was also carried out during cold storage. There are three observed parameters of spermatozoa speed: VCL, VSL and VAP. During cold storage 3-5°C, there was a trend of decreasing spermatozoa velocity (upper and lower layer) in all treatments. There was no interaction between diluents and antioxidants . In the upper layer (Table 3 and Table 4), VCL values (day 0 and 5), VSL (days 0) and VAP (days 0 and 5) of spermatozoa were significantly different between two diluents. The spermatozoa velocity in lower layer showed CEP-2 diluents was better than andromed at day 0 (VCL, VSL, VAP) and 5 (VSL, VAP).

A previous study by Penfold *et al.* (1998) stated that there is no difference between sperm X and Y in spermatozoa velocity. Many factors can influence spermatozoa velocity, which is viscocity of diluents, pH, energy sources, osmolarity (Perumal *et al.,* 2014). Viscosity of diluent can influence the capability of spermatozoa to penetrate albumin, but it was not observed in this research. The decreased of spermatozoa velocity in andromed diluents was faster than CEP-2. It was probably due to the less sugar (as an energy sources) composition of Andromed than CEP-2. Energy sources of CEP-2 were sorbitol, fructose and egg yolk.

Motility and velocity of spermatozoa are

Table 3. Velocity Spermatozoa in the Upper Layer (X)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
VCL (µm/s)	0	60.4	53.7	58.3	55.8	*	ns	ns
	5	34.4	29.8	31.2	32.9	*	ns	ns
VSL (µm/s)	0	21.2	17.1	19.0	19.4	*	ns	ns
	5	15.2	12.7	15.1	12.8	ns	ns	ns
VAP (µm/s)	0	35.4	30.5	33.1	32.8	*	ns	ns
	5	22.8	18.1	21.1	19.9	*	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; VCL= velocity curve linear; VSL= velocity straight linear; VAP= velocity average pathway; *= significant (P<0.05); ns= non significant (P>0.05)

Table 4. Velocity Spermatozoa in the Lower Layer (Y)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
VCL (µm/s)	0	57.1	50.5	54.2	53.4	*	ns	ns
	5	35.3	29.7	33.0	32.0	ns	ns	ns
VSL (µm/s)	0	19.2	17.2	18.6	17.8	*	ns	ns
	5	15.1	12.5	14.3	13.3	*	ns	ns
VAP (µm/s)	0	33.4	28.9	32.0	30.2	*	ns	ns
	5	23.0	18.2	21.1	20.2	*	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; VCL= velocity curve linear; VSL= velocity straight linear; VAP= velocity average pathway; *= significant (P<0.05); ns= non significant (P>0.05)

closely related to the availability of energy sources in the diluent. Decreasing of spermatozoa velocity is associated with the availability of energy sources which is decreases during cold storage, namely sorbitol and fructose. Previous studies by Perumal et al. (2014) stated that the parameters of Forward Progressive Motility (FPM) and Velocity (VCL, VSL, and VAP) are factors that greatly influence to the fertilization. Similarly, Kathiaravan *et al.* (2011) stated that parameter VCL, VSL and VAP have a big role to predict fertility by in vivo. The VCL is related to the sperm capacity to penetrate cervical mucus, meanwhile, VSL is directly related to fertility (Amal et al., 2019). Another factor that decreases velocity during storage was the production of ROS by damaging the membrane integrity of spermatozoa.

Malik *et al.* (2011), Hollinshead *et al.* (2004) and Blondin *et al.* (2009) stated that decreasing motility and velocity of sexed spermatozoa were caused by sperm sexing process. Treatment in sexing such as centrifugation, cold shock, osmotic and oxidative stress influences to the motility and velocity. After sperm sexing processed, spermatozoa need to adapting in new condition that different with before. It can be impacts on the decreasing of motility and spermatozoa membrane integrity.

Linearity (LIN), Straightness (STR) and Wobble (WOB)

The parameters of linearity, straightness and wobble, are parameters that indicate the spermatozoa swimming pattern. The observations of these three parameters on the upper (X) and lower (Y) spermatozoa during cold storage are listed in Table 5 and Table 6. Table 5 showed that the LIN, STR and WOB spermatozoa in the upper layers on day 5 showed significant difference between with and without glutathione addition. Spermatozoa in diluents added glutathione has higher value of LIN, STR and WOB which were showed progressive swimming patterns. Table 9 showed that swimming patterns of spermatozoa in the lower layers did not significantly difference between treatment, diluents (CEP-2 and andromed) and antioxidants on day 0 and 5 of cold storage. Spermatozoa in diluents added with glutathione showed higher LIN, STR, and WOB than without additional glutathione.

The previous study of Penfold *et al.* (1998) stated that there was a difference in swimming pattern (LIN, STR) between spermatozoa X and Y. The sexing process caused a decrease in LIN and WOB. Nevertheless, this study showed that antioxidant glutathione gives a positive impact to swimming patterns in the upper layer (Spermatozoa X). Ansari *et al.* (2014) and El-kon and Darwish. (2011) reported that glutathione supplementation in semen diluents can be limiting the damage of mammalian integrity membrane by ROS. Glutathione can improve sperm motility, acrosomal integrity, plasma membrane integrity, and viability during the freeze and thawing processes (Ansari *et al.*, 2014). Membrane integrity can support mitochondrial function for producing ATP, so that it can maintaining spermatozoa motility (Susilawati, 2011).

Amplitude Lateral Head (ALH) and Beat Cross Frequency (BCF)

Table 5. Linearity (LIN), Straighness and Wobble Spermatozoa in the Upper Layer (X)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
LIN (%)	0	35.4	33.2	33.0	35.5	ns	ns	ns
	5	44.1	43.1	48.6	38.6	ns	*	ns
STR (%)	0	60.2	57.1	57.7	59.6	ns	ns	ns
	5	66.8	69.9	72.2	64.5	ns	*	ns
WOB (%)	0	58.6	57.6	57.1	59.2	ns	ns	ns
	5	65.7	61.2	67.3	59.6	ns	*	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; LIN=linearity; STR=straightness; WOB=wobble; *= significant (P<0.05); ns= non significant (P>0.05)

Table 6. Linearity (LIN), Straightness and Wooble Spermatozoa in the Lower Layer (Y)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
LIN (%)	0	34.0	35.1	35.1	34.0	ns	ns	ns
	5	43.4	42.8	44.1	42.1	ns	ns	ns
STR (%)	0	57.9	60.3	58.6	59.6	ns	ns	ns
	5	66.2	68.7	68.5	66.4	ns	ns	ns
WOB (%)	0	58.6	57.8	59.4	56.9	ns	ns	ns
	5	65.5	62.0	64.2	63.3	ns	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; LIN=linearity; STR=straightness; WOB=wobble; *= significant (P<0.05); ns= non significant (P>0.05)

The ALH and BCF parameters showed the pattern of the spermatozoa tail movement. During cold storage, ALH and BCF values of spermatozoa in the upper and lower layers are listed in Table 7 and Table 8. In the upper layer, BCF of spermatozoa on day 0 by using CEP-2 was significantly higher than andromed, which were 10.4 and BCF 9.5 respectively. There was no significantly difference of ALH and BCF between treatment (diluents and antioxidants). The progressive motility, motility and BCF can be used as a standard for predicting in vivo bovine fertility (Oliveira *et al.*, 2013). The BCF values indicated the frequency of the spermatozoa movement through its average trajectory. The greater the BCF value indicates a stable and regular pattern of movement (not pretend to be hyperactive). Table 8 showed there was no difference between treatments on ALH and BCF. The ALH values indicate spermatozoa head deviations while swimming (Kathiravan *et al.*, 2011). The increasing ALH value indicates an inferior quality that can disturb the spermatozoa progression movement (Amal et al., 2019). The decreasing ALH value indicates that the spermatozoa are moving straighter and the speed is slower. Spermatozoa sperm sexing results in the upper and lower layers showed no difference between treatments and did not show increasing during cold storage. It was contrary to Tardif et al. (1996), which stated that ALH values sharply increasing after spermatozoa are cooled and spermatozoa tend to be more hyperactive during cold storage. Nevertheless, there was no clearly reasons of this condition (Tardif *et al.*, 1996).

Hyperactive Spermatozoa (H)

Another motility parameter is hyperactive spermatozoa. A high hyperactivity value is a good indication during fertilization, but it is a bad indication for the quality of fresh semen and semen during storage (cold or frozen). The observation of the hyperactive value of spermatozoa at day 0 in the upper and lower layer during cold storage showed that spermatozoa with CEP-2 diluents were higher than andromed. There was no significantly difference of spermatozoa hyperactive by the addition of glutathione (Table 9).

Ratnawati (2017) stated that spermatozoa hyperactivity is a condition where spermatozoa move very fast and strong but are less progressive and linear with an increase in lateral head amplitude (ALH). The hyperactivity value of spermatozoa is associated with high energy sources of CEP-2 at the beginning of storage which was fructose and sorbitol (Ratnawati, 2017). A low hyperactivity value indicates that spermatozoa can survive longer because there are still plenty of energy reserves and are not used to produce hyperactive movements. Hyperactive spermatozoa status can be identified through VCL, LIN, and ALH parameters. Spermatozoa become hyperactive when VCL values> 150µm/s, LIN <50% and ALH>5 µm (Ratnawati , 2017). Kathiaravan *et al.* (2011) stated that an indication of hyper-activated sperms is the greater value of VCL ≥70 µm /s and ALH ≥7µm. Spermatozoa

Table 7. Amplitude of Lateral Head and Beat Cross Frequency Spermatozoa in the Upper Layer (X)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
ALH (µm)	0	2.5	2.4	2.6	2.4	ns	ns	ns
	5	1.6	1.8	1.6	1.9	ns	ns	ns
BCF (Hz)	0	10.4	9.5	9.8	10.2	*	ns	ns
	5	9.6	9.3	9.7	9.3	ns	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; ALH=amplitude lateral head; BCF=beat cross frequency; *= significant (P<0.05); ns= non significant (P>0.05)

Table 8. Amplitude of Lateral Head and Beat Cross Frequency Spermatozoa in the Lower Layer (X)

Parameter	Day	Diluent		Glutation		Significance		
		CEP-2	Andromed	With	Without	D	G	INT
ALH (µm)	0	2.5	2.3	2.4	2.5	ns	ns	ns
	5	1.7	1.9	1.8	1.7	ns	ns	ns
BCF (Hz)	0	9.1	9.7	9.6	9.2	ns	ns	ns
	5	9.4	8.6	8.7	9.3	ns	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; ALH=amplitude lateral head; BCF=beat cross frequency; *= significant (P<0.05); ns= non significant (P>0.05

Table 9. Hyperactivate (H) Spermatozoa in the Upper Layer (X) and Lower Layer (Y)

Day	Diluent		Glutation		Significance		
	CEP-2	Andromed	With	Without	D	G	INT
Upper Layer							
0	11.1	6.0	9.1	7.9	*	ns	ns
5	2.6	2.1	2.2	2.6	ns	ns	ns
Lower Layer							
0	11.5	5.6	7.8	9.3	*	ns	ns
5	3.3	2.5	3.2	2.6	ns	ns	ns

CEP-2= Caudal Epididimis Plasma; D= Diluent; G= Glutation; INT= interaction between diluent and glutation; *= significant (P<0.05); ns= non significant (P>0.05)

will move quickly but are less linear and tend to make them move around (star-shaped pattern).

CONCLUSION

Sexed semen with CEP-2 diluents showed a better motility of spermatozoa than andromed in the upper layers (spermatozoa X) and lower layers (spermatozoa Y). The addition of Glutathione 1mM can support the motility of sexed -spermatozoa in cold storage, especially for LIN, STR, and WOB values. There was no interaction between diluents and glutathione on spermatozoa motility of sexed semen.

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