Viability and abnormality of sexed spermatozoa with albumin gradient in different diluents and antioxidants treatment

D Ratnawati and F Firdaus
Indonesian Beef Cattle Research Station, Pahlawan Street. No. 2 Grati, Pasuruan, East Java, Indonesia 67184
E-mail: dian_sapo@yahoo.co.id

Abstract. Livestock is an agricultural sub-sector that contributes sufficiently to the needs of protein. One of the bio reproduction technologies to support livestock productivity is sexing technology. The purpose of this study was to measure the viability and abnormality of sexed spermatozoa from sexing with albumin with the treatment of diluents and antioxidants. The material used in this study was fresh semen of 5 PO bulls with progressive motility >70%. The sexing methodology used is 5%, 10%, and 15% albumin (egg white) gradients. The diluent treatment used was CEP-2 and andromed, with the addition or without the 1mM antioxidant (glutation). The sexed semen was made in liquid semen and stored at 3-5 °C. Sample preparation was done by making a smear of spermatozoa on glass objects and colored with eosin negrosin. Viability and abnormality analysis was performed on days 0 (H0) and 5 (H5). Viability and abnormality analysis of spermatozoa using SCA v.2.1. Parameters measured included: spermatozoa viability and abnormality. The design of the experiment used a 2x2 factorial pattern. Data analyzed with SPSS 24. The viability of spermatozoa from sexing using egg white albumin in andromed diluents is better than CEP-2 on storage days 0-2. Abnormalities of spermatozoa of sexing during cold storage were not influenced by diluents (CEP-2 and andromed) or the use of glutathione. The viability of spermatozoa during cold storage (>80%) and abnormalities (<20%) indicate the quality of liquid semen results from sex is still good.

1. Introduction
Livestock is an agricultural sub-sector that contributes sufficiently to the needs of protein. One of the bio reproduction technologies to support livestock productivity is sexing technology. Sexing using albumin is a relatively easy method. The principle working is based on the motility of the spermatozoa. It is known that X spermatozoa (females) have a large head size so that their motility is lower, and they are less able to penetrate albumin (upper layer). Meanwhile, Y spermatozoa with a small head size allow more movement to penetrate albumin (lower layer). Several previous studies have conducted sexing using stratified albumin concentrations including 30% and 10%; 50%, 30%, and 10%. In 2018, Beef Cattle Research conducted a sexing study using a 5%, 10%, and 15% albumin gradient with CEP-2 as diluents.

The main parameters of semen analysis to determine male bull fertility are sperm motility and morphology of spermatozoa [1]. Spermatozoa motility is a basic parameter that is simple, fast, cheap, and commonly used to determine semen quality for bulls [2]. The percentage of normal spermatozoa’s value is similar to the percentage of progressively motile spermatozoa. Normal sperm significantly affects the fertility of bulls, and the rate of sperm abnormalities affects decreasing fertility. The
morphology and structure of spermatozoa have an important role in successful fertilization, early embryo development, and the level of pregnancy. Abnormality spermatozoa are closely related to the incidence of chromosomal structure abnormalities, chromosomal abnormalities, unstable chromosomes, and low DNA quality [3,4].

Spermatozoa morphology consists of 2 parts, namely the head and tail. The head consists of the anterior acrosome and the posterior acrosome. The anterior acrosome covered by an acrosome covering consisting of hormones that play an important role in the fertilization process, including are acrosine, hyaluronidase, and other hydrolytic enzymes. The tail consists of 3 main parts, namely the midpiece, principal piece, and end piece. Spermatozoa abnormalities, according to [1] divided into primary (occurring during spermatogenesis) and secondary (occurring after spermiation). Based on the location of the damage to the sperm, it can be divided into primary (abnormalities in the spermatozoa’s head), secondary (abnormalities in the spermatozoa’s mid piece) and tertiary (abnormalities in the spermatozoa’s tail) [5].

This study aimed to measure the viability and abnormality of sexed spermatozoa (X and Y) by using the albumin method, with different diluents (CEP-2 and andromed) and the use of antioxidants.

2. Materials and methods
The Animal Committee approved the Ministry of Agriculture research with the registration number Balitbangtan /Lolitsapi /Rm /01/2018. The material used was fresh semen of PO bulls with 10 replications (semen storage). The research was conducted in the cages and animal reproduction laboratory of the Beef Cattle Research Station. The study was conducted in July-October 2018. Collecting semen was carried out by using an artificial vagina. The fresh semen requirement of PO bulls has a progressive motility >70%. The sexing procedure was performed using an albumin gradient of 5%, 10%, and 15%. The treatments included diluents (CEP-2 and andromed) and antioxidants (glutathione and without glutathione). The sexed semen was processed into liquid semen and stored at 3-5 °C until day 5.

2.1. Assessment of spermatozoa viability
The preparation began by mixing the sexed semen, followed by a drop of mixture with one drop of eosin negrosin stain and smear on a glass object. The next step was fixing the glass object with fire so that the spermatozoa can adhere and last a long time on the glass object. The prepared glass object was examined on a SCA v.2.1 microscopes with magnification 1000 times. Live spermatozoa will appear white, while dead spermatozoa will appear red. The observations made in one field of view or up to 100 spermatozoa. The viability value is expressed in percentage. The calculation method was as followed:

\[
\text{Number of live spermatozoa x 100% } \\
\text{The number of spermatozoa observed (live and dead)}
\]

2.2. Assessment of spermatozoa abnormalities
The assessment of spermatozoa abnormality was done by using the same spermatozoa smear that use to analyze spermatozoa viability. Identification of the type of abnormality spermatozoa was carried out using SCA v.2.1. on 100 spermatozoa. The value of abnormal spermatozoa was expressed in percentage. The calculation method was as followed:

\[
\text{Number of abnormal spermatozoa x 100% } \\
\text{The number of spermatozoa observed (normal and abnormal)}
\]

2.3. Data analysis
The experimental design was a 2x2 factorial pattern, the first factor was the type of diluents (CEP-2 and andromed), and the second factor was the use of antioxidants (glutathione and without glutathione). Data analyzed by using the general linear model (GLM), IBM SPSS 24.
3. Results and discussion
Observation of spermatozoa viability and abnormality was carried out during days 0-5 at cold storage at 3-5 °C.

3.1. Viability and abnormalities of sexed spermatozoa
Observation of spermatozoa was done on the upper layer (Spermatozoa X) (table 1) and the lower layer (Spermatozoa Y) (table 2).

### Table 1. Viability and abnormalities of sexed spermatozoa (X) on the upper layer.

| Parameter | Day | Diluents | Sign. | Glutation | Sign. | Int. |
|-----------|-----|----------|-------|-----------|-------|------|
|           |     | CEP      | Andromed | Without Glutation | With Glutation |
| Viability (%) | 0   | 89.7     | 95.0   | <0.05     | 92.5 | 92.2 | >0.05 | >0.05 |
|           | 1   | 89.6     | 95.2   | <0.05     | 90.3 | 94.5 | <0.05 | >0.05 |
|           | 2   | 89.3     | 94.8   | >0.05     | 92.5 | 91.7 | >0.05 | >0.05 |
|           | 3   | 90.7     | 93.8   | >0.05     | 92.3 | 92.2 | >0.05 | >0.05 |
|           | 4   | 89.8     | 93.3   | >0.05     | 91.8 | 91.3 | >0.05 | >0.05 |
|           | 5   | 88.3     | 89.3   | >0.05     | 91.1 | 86.4 | >0.05 | <0.05 |
| Abnormality (%) | 0   | 1.2      | 0.3    | >0.05     | 1.0  | 0.5  | >0.05 | >0.05 |
|           | 1   | 0.9      | 1.0    | >0.05     | 1.1  | 0.8  | >0.05 | >0.05 |
|           | 2   | 0.6      | 1.2    | >0.05     | 0.8  | 0.9  | >0.05 | >0.05 |
|           | 3   | 0.2      | 0.0    | >0.05     | 0.0  | 0.2  | >0.05 | >0.05 |
|           | 4   | 0.8      | 0.8    | >0.05     | 0.9  | 0.7  | >0.05 | >0.05 |
|           | 5   | 1.4      | 2.0    | >0.05     | 1.6  | 1.8  | >0.05 | >0.05 |

Note: CG (CEP-2 + Glutathione), C (CEP-2), AG (Andromed + Glutathione), A (Andromed), D (Day), Sign (Significantly), Int (Interaction).

During cold storage, it was known that viability spermatozoa in andromed showed significantly higher than CEP at cold storage on days 0 and 1. The use of glutathione at storage (day 1) showed a significantly lower percentage of viability spermatoza. There is a significant interaction between the type of diluent and the use of antioxidants on day 5 (H5) of cold storage. Meanwhile, there was no significant difference (P> 0.05) of spermatozoa abnormality between diluents (CEP and andromed) and the use of antioxidants (glutathione) during cold storage. There was no interaction between the type of diluent and the addition of glutathione to the semen during cold storage (D1-D5). At the lower layer, the viability and normality of spermatozoa are presented in table 2.

At the lower layer, the viability of spermatozoa in andromed at day 2 (D2) has a significantly higher percentage than CEP. There is an interaction between the use of diluents and the addition of glutathione on day 4 (D4) cold storage. Meanwhile, there was no significant difference between using different diluents (CEP and andromed) and antioxidants (glutathione) during cold storage on abnormal spermatozoa. There was no interaction between the type of diluent and the addition of glutathione to the semen during cold storage (D1-D5).

Overall, the viability value above 80% indicates the quality of the sexed semen was appropriate to the standard of semen quality. Likewise, the abnormality value of spermatozoa during cold storage is still <20% [6], so that it was appropriate to the standard of semen quality. The composition of Andromed includes tris aminomethane, citric acid, glycerol, fructose, and phospholipids. Andromed also contained vegetable lecithin, which functions to protect the plasma membrane of spermatozoa. Glycerol functions as an intracellular cryoprotectant, which also plays a role in protecting the integrity of the membrane. The composition of CEP-2, egg yolk containing lipoproteins and phospholipids, has an important role as an extracellular cryoprotectant and maintains the integrity of the spermatozoa.
membrane [7]. The andromed and CEP-2 have the same performance in maintaining the viability of sexed spermatozoa [8].

Table 2. Viability and abnormalities of sexed spermatozoa (Y) at lower layer.

| Parameter   | Day | Diluents | Sign. | Glutation | Sign | Int. |
|-------------|-----|----------|-------|-----------|------|------|
|             |     | CEP      | Andromed | With | Without | With |
| Viability (%) | 0   | 90.7     | 95.0   | >0.05   | 92.5 | 93.2 | >0.05 | >0.05 |
|             | 1   | 89.1     | 93.3   | >0.05   | 90.2 | 92.3 | >0.05 | >0.05 |
|             | 2   | 86.0     | 95.8   | <0.05   | 90.2 | 91.7 | >0.05 | >0.05 |
|             | 3   | 87.2     | 91.7   | >0.05   | 88.0 | 90.8 | >0.05 | >0.05 |
|             | 4   | 88.3     | 91.5   | >0.05   | 92.0 | 87.8 | >0.05 | <0.05 |
|             | 5   | 85.4     | 88.8   | >0.05   | 90.4 | 83.8 | >0.05 | >0.05 |
| Abnormality (%) | 0   | 1.3      | 0.8    | >0.05   | 1.2  | 1.0  | >0.05 | >0.05 |
|             | 1   | 0.5      | 0.8    | >0.05   | 0.8  | 0.6  | >0.05 | >0.05 |
|             | 2   | 0.7      | 0.7    | >0.05   | 0.7  | 0.7  | >0.05 | >0.05 |
|             | 3   | 0.8      | 0.7    | >0.05   | 0.9  | 0.5  | >0.05 | >0.05 |
|             | 4   | 1.1      | 0.3    | >0.05   | 0.5  | 0.8  | >0.05 | >0.05 |
|             | 5   | 1.1      | 1.8    | >0.05   | 1.5  | 1.4  | >0.05 | >0.05 |

Note: CG (CEP-2 + Glutathione), C (CEP-2), AG (Andromed + Glutathione), A (Andromed), D (Day), Sign (Significantly), Int (Interaction).

The sperm morphology score contributed 40% to the total BSE score. Low sperm morphological values are usually the main cause of old bulls [9]. There is a clear relationship between abnormal spermatozoa morphology and poor DNA quality. Primary spermatozoa abnormalities mostly caused by genetic factors, resulting in failed apoptosis due to damaged DNA. Morphologically, abnormal spermatozoa are also susceptible to DNA damage [3]. The occurrence of abnormal spermatozoa indicates a pathological process in the reproductive tract of bulls (testes, epididymis, or accessory gland) or an indication of decreasing semen fertility [10]. Abnormalities of spermatozoa in the upper layer (X) and the lower layer with andromed diluent and CEP-2 showed the same performance; this is consistent with the previous research results [8].

Two main factors influence sperm abnormalities, including endogenous and exogenous factors. The endogenous factors are age, sperm maturation, energy reserves, and active agents (surface). Exogenous factors that influence include physiology and biophysics, suspending fluids, stimulation-inhibition. The things that need to be considered in the morphology of spermatozoa are the head, middle, and tail. Abnormalities in the head have an impact on livestock fertility because they are related to genetic material. Meanwhile, the abnormality in the tail impacts decreasing the motility of spermatozoa so that they cannot move progressively.

3.2. Observation of membrane integrity with SEM (Scanning Electron Microscope)
Observation of the membrane integrity of spermatozoa was carried out by Scanning Electron Microscope (SEM) analysis, which was conducted at the Central Laboratory of UM, State University of Malang. The samples observed were spermatozoa samples during cold storage (D0, D3, D6). Visualization of sexed spermatozoa in the upper and lower layers presented in figures 1, 2, and 3.
Figure 1. Spermatozoa at day 0 storage (magnification 5,000 and 10,000 times).

Figure 2. Spermatozoa at day 3 storage (magnification 5,000 and 10,000 times).
Figure 3. Spermatozoa at day 6 storage (magnification 5,000 and 10,000 times).

The observation use SEM with the magnification of 5,000 times and 10,000 times. The picture showed that spermatozoa during cold storage at D0, D3, and D6 in the upper and lower layers did not show membrane damage. Overall, the spermatozoa head membrane was still intact. The condition of the spermatozoa membrane integrity indicates that the situation during cold storage is very conducive. The excellence of diluents performance can protect the spermatozoa membrane from damage caused by ROS (reactive oxygen species). The cryoprotectants content in the diluents was able to maintain the spermatozoa membrane. Spermatozoa with incompleted or damaged membranes indicate a non-motile condition. The two main factors determine the ability of spermatozoa to fertilize an ovum. The integrity of the membrane has a major role in forming bonds with the zona pellucida. In contrast, the second role is spermatozoa motility in the effort to penetrate cumulus and zona pellucida.

4. Conclusions
The viability of sexed spermatozoa using albumin in andromed diluents was better than CEP-2 at storage days 0-2. The abnormality of sexed spermatozoa during cold storage was almost not influenced by diluents (CEP-2 and andromed) or the use of glutathione. Spermatozoa viability during cold storage > 80% and abnormality <20% indicate that the quality of the liquid sexed semen is appropriated to the standard.

References
[1] Corbet N J et al 2013 Male traits and herd reproductive capability in tropical beef cattle genetic parameters of bull traits Anim. Prod. Sci. 53 (2) 101–113
[2] Gliozzi T M, Turri F, Manes S, Cassinelli C and Pizzi F 2017 The combination of kinetic and flow cytometric semen parameters as a tool to predict fertility in cryopreserved bull semen Animal 11 (11) 1–8
[3] Enciso M, Cisale H, Johnston S D, Sarasa J, Fernández J L and Gosálvez J 2011 Major morphological sperm abnormalities in the bull are related to sperm DNA damage
Theriogenology 76 (1) 23–32

[4] Morrell J M, Valeanu A S, Lundeheim N and Johannisson A 2018 Sperm quality in frozen beef and dairy bull semen Acta Vet. Scand. 60 (1) 1–11

[5] Purwantara B, Arifiantini R I and Riyadhi M 2010 Sperm morphological assessments of Friesian Holstein bull semen collected from three Artificial Insemination centers in Indonesia J. Indones. Trop. Anim. Agric. 35 (2) 90–94

[6] Afiati F 2004 Proporsi dan karakteristik spermatozoa X dan Y hasil separasi kolom albumin Media Peternak. 27 (1) 14–17.

[7] Lele Y U Kusumawati E D and Krisnaningsih A T N 2017 Motilitas dan viabilitas spermatozoa semen sexing kambing peranakan etawa (pe) menggunakan metode sedimentasi putih telur dengan pengencer yang berbeda J. Sains Peternak. 5 (1) 50–56.

[8] Ervandi M Susilawati T and Wahyuningsih S 2013 Pengaruh pengencer yang berbeda terhadap kualitas spermatozoa sapi hasil sexing dengan gradien albumin (putih telur) J. Ilmu Ternak dan Vet. 18 (3) 177–184

[9] Godfrey R W and Dodson R E 2005 Breeding soundness evaluations of Senepol bulls in the US Virgin Islands Theriogenology 63 (3) 831–840

[10] Al-Makhzoomi A Lundeheim N Håård M and Rodríguez-Martínez H 2008 Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden Theriogenology 70 (4) 682–691