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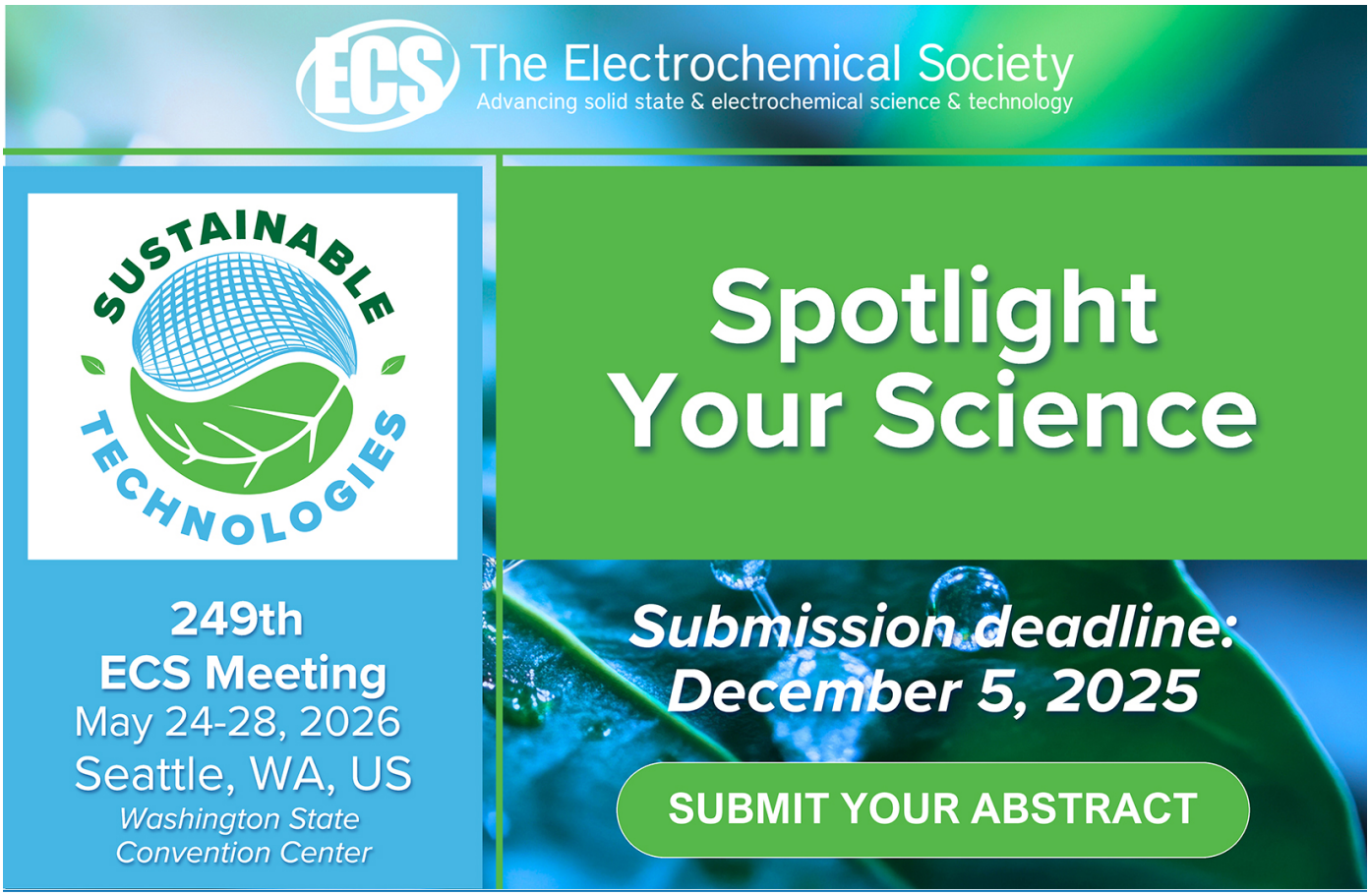
To cite this article: A Rachmawati *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **478** 012068



Effect of Different Bovine Serum Albumin (BSA) Levels on the Sperm Viability of Ongole Cross Bred Bull during 5°C Storage

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Abstract. Improving the productivity and genetic quality of local cattle, especially Ongole Cross Bred (known as PO) is applied through Artificial Insemination (AI). The success of AI must be supported by semen quality. Semen processing at 5°C can cause sperm membrane damage resulting in decreased semen quality, especially the viability of sperm. This results in reduced sperm fertility at the time of fertilization with the ovum. Viability is an important indicator for cold semen processing. The use of semen diluents, Cauda Epididymal Plasma-2 (CEP-2), has been shown to be able to maintain semen quality during storage at 5°C. The addition of BSA as an extracellular cryoprotectant supported the function of egg yolk in CEP-2 to prevent cold shock during storage. This study used fresh ejaculate of PO bull with a minimum requirement of 80% viability. The ejaculate was diluted by CEP-2 with BSA level 0; 0.2; 0.4; 0.6; 0.8 and 1%. The diluted ejaculate was stored for eight days at 5°C. The results showed that the viability of sperm fluctuated in line with the length of storage at each level of BSA. Significant influence of BSA on sperm viability occurred at storage day 4 (83.29±3.53)% at 1% level and day 8 (89.36±2.65)% at 0.2 % level ($P < 0.01$). The conclusion was the BSA 0.2% level maintains the highest viability of sperm during eight days of storage. Suggestion for further research was the need for research on motility and abnormality as a support for the successful processing of liquid semen.

1. Introduction

Crosses between local cows with superior Ongole bulls produced Ongole Cross Bred (known as PO) or called Ongolisasi, which began since the Dutch Indies Government [1]. Ongole Cross Bred cattle provide a significant contribution to animal protein food needs in Indonesia. The existence of PO cattle as national germplasm must be maintained to be productive. The advantages of PO cattle are that they have high adaptability to hot tropical environments, have reliable energy and cow reproductive activities after breeding. Bull PO have good semen quality [2].

To support the Ongolization Program in East Java Province [1] and the Special Effort for Obligatory Bovine Cows (UPSUS SIWAB) to accelerate the target fulfilment of domestic beef cattle populations and Meat Self-Sufficiency in 2026, namely increasing population and genetic quality of PO cattle through Biotechnology of Artificial Insemination (AI) and Intensification of Natural Mating (Inka). The program is outlined in the Minister of Agriculture Regulation Number: 48/Permentan/PK.210/10/2016 concerning Special Efforts to Accelerate the Increased Population of Cattle and Buffaloes signed by the Minister of Agriculture on October 3, 2016. Artificial Insemination will be successful if supported by

quality semen. Liquid semen technology is widely applied in AI, especially in areas where liquid nitrogen is difficult.

Semen that is cooled (liquid semen) and diluted is an excellent alternative to frozen semen when used for a short time after storage. Freezing and thawing cause damage to spermatozoa, freeze-thawed semen generally records more reduced quality and fertility, compared to diluted or fresh liquid semen. The quality and viability of spermatozoa during cold storage gradually deteriorate because of the storage time increases [3].

The processing of liquid semen must be supported by diluents which can maintain the quality of the cement during 5^{o}C storage. Semen dilution aims to increase the volume of each ejaculate and provide the nutrients needed to maintain sperm viability and fertility, provide protection against sperm against cold shocks, prevent the development of microorganisms and as a buffer in maintaining pH stability (buffering) [4] [5]. Diluents must have properties such as seminal plasma that can create conditions for spermatozoa to survive in artificial conditions during the storage process. Viability is an essential factor in the processing of liquid semen because viability affects the fertility of spermatozoa during fertilisation with oocytes.

Cauda Epididymal Plasma-2 (CEP-2) diluents have the same chemical composition, such as NaCl, KCl, $CaCl_2(H_2O)_6$, NaH_2PO_4, KH_2PO_4, fructose, sorbitol, tris, gentamicin, citric acid and osmolality which are the same as the seminal plasma in the cauda epididymis [6]. CEP-2 do not contain antioxidants that protect sperm from free radicals or Reactive Oxygen Species (ROS) that cause damage to DNA, carbohydrates, lipids, nucleic acids and proteins in the nucleus and cell membranes [7]. Semen processing, such as storage at 5^{o}C, membrane damage results in the death of sperm by more than 50% which decreases motility or the ability of sperm to move progressively [8] [9].

The addition of albumin, such as BSA, as an extracellular membrane in CEP-2 diluents, is expected to be able to maintain membrane function related to sperm quality. The BSA content in CEP-2 is a macromolecule that acts to bind Ca^{2+} ions, prevents the entry of excessive Ca^{2+} ions into the cytosol, allows the membrane to more effectively regulate the movement of Ca^{2+} ions across the membrane and inhibits the accumulation of intracellular Ca^{2+} ions to toxic levels for sperm, so that viability, motility and the number of uncapacitated spermatozoa can be maintained high [10]. The viability of Piedmontese sperm was (38.74±0.854)% after storage at 4^{o}C for 31 days with a BSA level of 8 mg/ml. The BSA levels used were 0, 1, 4, 8, 12, and 16 mg/ml BSA in BIOXcell® Diluents (IMV Technologies, L'Agile, France) [11].

Supplementation of 20% egg yolk in CEP-2 diluents was able to maintain the motility of Limousin sperm (44.25±3.92)% and viability of (87.46±5.40)% during eight-day storage at temperatures 4-5°C [12]. Research on semen quality, especially viability, has not been done much with CEP-2 diluents with BSA as extracellular membranes, especially on PO sperm, so research on the role of different BSA levels on the viability of liquid semen in CEP-2 diluents is needed.

2. Material and Methods

2.1. Material

The material used for this research was ejaculate or fresh semen PO bull which were kept in the Beef Cattle Research Station, Grati District, Pasuruan Regency, as many as three heads aged three years, body weight ranges from 422-488 kg, and ejaculate was accommodated once a week. Bulls were placed in individual stall and clinically healthy. Bulls were fed in the form of concentrates (KUTT Sukamakmur, Grati Subdistrict, Pasuruan Regency), *Indigofera spicata*, elephant grass and straw, while drinking water was given ad libitum. Estrus cows were used as a teaser. The chemicals used for processing liquid semen were CEP-2+10% egg yolk diluents with BSA level 0; 0.2; 0.4; 0.6; 0.8 and 1%, eosin-negrosin staining and physiological NaCl 3%.

2.2. Methods

2.2.1. Ejaculate collection
The bulls were cleaned all part of stomach and prepuce to avoid contamination which had a negative impact on the quality of ejaculate and sperm. Ejaculate collection used the artificial vaginal method.

2.2.2. Viability assessment
Assess the percentage of sperm viability by dripping one or two drops of semen on the edge of the glass object and dropping one or two drops of eosin-negrosin staining on the precipitated semen. Semen and eosin-negrosin dyes were carefully homogenised using ose. The mixture of semen and staining was pushed to the end of the glass object using another glass object with a slope of 45° and dried at room temperature. Observations were made on 200 sperm using a 400X magnification light microscope. Dead sperm absorbed colour (purple or pink), because the membrane was unstable, whereas live spermatozoa were transparent. Viability calculation was done by finding the proportion of sperm that absorb and transparent as a percentage [13] [14].

$$\% \text{ viability} = \frac{\text{number of live sperm}}{\text{number of sperm observed}} \text{ x } 100\%$$

2.2.3. Semen diluting
Semen dilution was carried out at room temperature, with CEP-2+10% egg yolk with BSA level 0; 0.2; 0.4; 0.6; 0.8 and 1%. 0.5 ml fresh semen or ejaculate was added diluent with a different BSA level at 37°C (semen was put in a 15 ml test tube and soaked in a water jacket) as much as 1: 1 (diluent A1). If the temperature of the semen reached 30, 25, 20 and 12°C, the semen was added with diluent A2. The semen was incubated at 20°C for 15 minutes, and a viability examination was performed with a smear preparation.

2.2.4. Storage at 5°C
Semen was added with diluent in a 15 ml test tube, and semen samples in each treatment had been stored at 5°C for eight days. If the temperature of the semen reached 5°C, viability test was performed. Viability tests were observed using a 400x magnification light microscope.

2.3. Observation variables
The best BSA level and sperm viability during eight days of storage at 5°C.

2.4. Data analysis
Viability data were analysed using ANOVA Repeated Measured with a Completely Randomized Design. If there was a difference between treatments, then followed by Duncan's multiple range tests.

3. Result and Discussion

3.1. Sperm viability on fresh ejaculate
Viability of sperm can be determined by observing smear preparations stained with eosin-negrosin staining. Determination of viability by comparing the number of live sperm (white and does not absorb colour) and dead sperm (absorbing eosin-negrosin color), because the spermatozoa membrane is unstable. Viability of sperm in fresh semen for processing liquid or frozen semen is at least 80% [15]. Viability of sperm in fresh semen of research result was (92.59$\pm$4.15)%, and these result was a reference that fresh semen was suitable for processing liquid semen.

3.2. Sperm viability during freezing process

The results of the smear viability of sperm used eosin-negrosin staining and the colour difference between dead and living sperm can be seen in Figure 1. Differences in the affinity of living and dead sperm membranes in absorbing eosin-negrosin staining caused by the permeability of dead and live sperm. The membrane was unstable; then dead spermatozoa quickly absorb the colour. The results of the PO sperm viability examination had been storage at 5°C for eight days (Table 1).

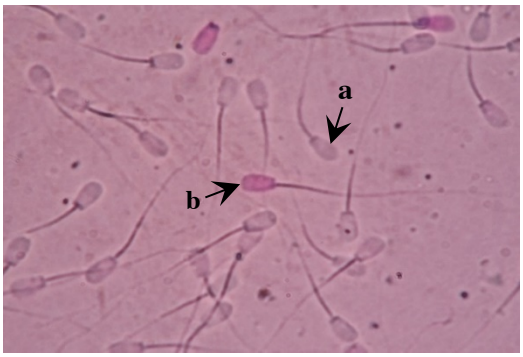

Figure 1. Observation of spermatozoa viability using eosin-negrosin staining and observed with light microscope (Olympus CX-21, Japan) 400x magnification. Note: a. lives or viable (transparent) spermatozoa and b. spermatozoa die (absorb color).

Table 1. Viability of PO Sperm with Different BSA Levels in CEP-2 Diluents

BSA Level (%)	Storage Time (day)								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
0	86.33±3.80^{a}	88.63±9.48^{a}	85.50±4.96^{a}	83.85±2.77^{ab}	74.46±5.12^{b}	73.70±6.20^{abc}	82.65±4.21^{a}	86.17±3.38^{a}	86.46±2.04^{a}
0.2	84.73±8.23^{a}	88.68±5.57^{a}	76.73±14.82^{b}	84.67±2.67^{ab}	79.39±4.51^{a}	70.49±4.10^{bc}	82.84±2.83^{b}	80.06±5.63^{b}	89.36±2.65^{a}
0.4	85.09±3.77^{a}	86.98±7.06^{a}	80.17±12.39^{ab}	80.54±2.38^{c}	71.65±5.60^{b}	72.17±5.87^{bc}	81.36±3.38^{ab}	83.28±2.53^{ab}	81.78±4.82^{b}
0.6	86.87±6.45^{a}	83.95±12.56^{a}	85.37±4.04^{a}	86.15±5.98^{a}	83.27±5.04^{a}	66.77±11.41^{c}	84.65±3.03^{ab}	83.33±5.81^{ab}	81.77±5.11^{b}
0.8	85.27±3.49^{a}	86.44±7.64^{a}	79.73±7.03^{b}	82.39±2.43^{bc}	81.88±2.27^{a}	80.56±5.06^{a}	83.84±4.00^{b}	81.39±2.40^{b}	76.18±6.35^{c}
1	83.45±4.42^{a}	88.62±7.87^{a}	81.36±6.38^{ab}	86.11±3.43^{a}	83.29±3.53^{a}	76.08±13.14^{ab}	82.32±4.19^{b}	80.77±6.22^{b}	79.40±4.55^{bc}
p value	0.761	0.800	0.241	0.004	<0.001	0.013	0.421	0.053	<0.001

Note : Different notation in the same column and row showed that the treatment had a significantly different effect ($p<0.05$) on the viability of sperm.

Based on the results of the study showed that different BSA levels in CEP-2 diluents were able to maintain the viability of spermatozoa for eight days of storage at 5°C. The fluctuating decrease in sperm viability during cold storage was caused by electrical voltage instability, ingredients contained in CEP-2 diluents, egg yolks and storage time. Changes in temperature and duration of storage affect the physical and chemical conditions of spermatozoa, although they were supported by the use of diluents that act as protectors and suppliers of nutritional needs. The significant influence of BSA on sperm viability occurred at storage day 4 (83.29±3.53)% at BSA level 1% and day 8 (89.36±2.65)% at BSA level 0.2% ($p<0.05$). Decrease and change in viability during storage was influenced by the condition of spermatozoa membrane phospholipids that are permanently damaged, thereby reducing the function of spermatozoa membranes and influencing the ability of spermatozoa to fertilise the ovum [12] [16]. Further research needs to be done, especially sperm motility and abnormality data, to support commercial production of liquid semen.

4. Conclusion

Level of BSA 0.2% level maintains the highest viability of sperm during eight days of storage (89,36±2,65)% following Indonesian National Standards.

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