# Sperm quality profile of Bali bull as local Indonesian cattle in liquid storage in different diluents with the addition of synthetic antioxidants

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**Abstract.** Bali cattle are one of Indonesia's local cattle, which require efforts to preserve genetic material from superior males by storing sperm at low temperatures so that it does not become extinct and can be implemented for artificial insemination. The simplest storage method is liquid storage, at 4-5°C. This study aimed to examine the quality profile of Bali bull sperm during storage at 4-5°C in CEP diluent with and without synthetic antioxidants (alpha-tocopherol, glutathione) and to compare it with the diluent media usually used by Indonesian Artificial Insemination centers for freezing bovine semen, namely tris aminomethane egg yolk. The research used 3 Balinese bull ejaculates, each replicated 3 times. Fresh semen was collected using an artificial vagina. The sperm quality profile included motility, viability, and membrane integrity a. Sperm motility was observed using the CASA IVOS 2 tool. Sperm viability was observed using the eosin nigrosine staining method under a microscope with 200 magnification. Membrane integrity was observed utilizing the HOST test (Hypo Osmotic Swelling Test). The results showed that the percentage of motility from the first day to the last day of storage was highest in CEP diluent with the addition of alpha-tocopherol and glutathione. The observations of membrane viability and integrity showed the highest percentage of CEP diluent with the addition of alpha-tocopherol from the first to the last day of storage. The research results show that Bali cattle sperm can be stored at low temperatures, especially in CEP diluent, with the addition of antioxidants.

## 1 Introduction

Bali cattle are one of Indonesia's native breeds. They were domesticated from wild banteng, so their phenotypic characteristics are similar to those of bulls [1]. Balinese cattle's advantages include surviving in a tropical environment with low-quality feed and performing well [2].

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Balinese cattle must be maintained and increased in population because the population will continue to decline if farmers prefer imported cattle. Efforts can be made to maintain and increase the population by applying reproductive biotechnology, namely Artificial Insemination (AI). AI usually uses sperm that has gone through dilution with diluent media and stored at a low temperature (4-5°C or freezing temperature). This sperm storage technology helps support the implementation of AI and maintain the genetic material of males so that it does not become extinct, especially for local native animals such as Balinese cattle.

The dilution media must maintain sperm quality during storage at low temperatures. One of the diluent media for bovine sperm storage is CEP diluent. The characteristics of CEP diluent are similar to the physical and chemical conditions that exist in the reproductive tract of bulls, namely cauda epididymal plasma, where in this channel, sperm is temporarily stored for the maturation process so that it can move quickly [3,4]. This study used CEP diluent media to store Bali cattle sperm with the addition of synthetic antioxidants alpha-tocopherol and glutathione. Antioxidants can suppress membrane damage due to free radical activity [5]. Antioxidants added to the diluent aim to prevent damage to the plasma membrane caused by Lipid peroxidation (LPO) [6].

The purpose of this study was to determine the shelf life of Bali Cow sperm at 4-5°C in CEP diluent with the addition of different synthetic antioxidants, namely alpha-tocopherol and glutathione, and compared to tris aminomethane diluent which is usually used at the Artificial Insemination Centre, Singosari, Malang, East Java, Indonesia.

## 2 Material and methods

### 2.1 Prepare CEP diluent and supplement synthetic antioxidants.

CEP was prepared from a mixture of NaCl 15 mmol/L; KCl 7.0 mmol/L; CaCl2(H2O)2 3.0 mmol/L; MgCl2(H2O)6 3.0 dmmol/L; NaHCO3 11.9 mmol/L; NaH2PO4 8.0 mmol/L; KH2PO4 20.0 mmol/L; fructose 55 mmol/L; sorbitol 1.0 gr/L; BSA 2.0 gr/L; Tris 133.7 mmol/L; penicillin 1000 IUI; streptomycin 1 gr; and citrate acid 42.6 mmol/L (Bioworld, USA), by the aliquot technique. CEP media is divided into four groups, namely P1 (CEP only), P2 (CEP + 2 mM alpha-tocopherol), P3 (CEP + 0,75 mM glutathione), dan P4 (CEP + 2 mM alpha-tocopherol + 0,75 mM glutathione). In comparison, the fifth group (P5) used tris aminomethane diluent media, which BBIB Singosari usually uses diluent media [3,4,7].

### 2.2 Semen collection

Semen collection of Balinese cows was conducted using an artificial vagina method conditioned at 42°C and added vaseline as a lubricant. An adult female cow was used as an attractant to increase male libido.

## 2.3 Semen processing

Semen was evaluated macroscopically and microscopically. The macroscopic evaluation included volume, color, odor, consistency, and pH. Microscopic evaluation included mass motility, individual motility, membrane integrity, viability, and spermatozoa concentration. Fresh semen that can be stored must have a viability percentage of≥ 70% and a motility percentage of ≥ 55%. Fresh semen was diluted using the prepared diluent media. Dilution was done at 37°C; then, the sample was stored in a refrigerator at 4-5°C. After the temperature dropped to 5°C, semen quality testing was continued as day 0, including motility, viability, and membrane integrity. The motility, viability, and membrane integrity were evaluated until day 3, while the DNA profile test was carried out on day 0 and day 3.

### *2.3.1 Sperm motility*

Evaluation of sperm motility was done objectively with the help of Computer-Assisted Semen Analysis (CASA) by looking at the progressive movement of spermatozoa motility in five fields of view.

### *2.3.2 Sperm viability*

Evaluation of sperm viability by the eosin nigrosine staining method. Semen samples were taken, dripped on the tip of the object glass, and mixed with eosin nigrosine dye. Then, a thin smear was observed under a microscope at 400x magnification [8]. A purplish-red spermatozoa head indicates that the spermatozoa are dead, while an unstained spermatozoa head suggests that the spermatozoa are alive.

### *2.3.3 Integrity membrane of sperm*

Evaluation of sperm membrane integrity by a Hypo Osmotic Swelling Test (HOST) solution consisting of 1.3 g fructose and 0.7 trisodium citrate dehydrate in 100 ml distilled water. Semen was mixed into the HOST solution and then incubated at 37°C warm water for 30 minutes. Observations were made by making thin smears of incubated samples and observed using a 400x magnification microscope. A circular spermatozoa tail indicates that the plasma membrane is still intact, while a straight tail suggests that the spermatozoa plasma membrane has been damaged [9].

## 2.4 Statistic Analysis

To compare five treatments in motility, viability, and membrane integrity of Bali Cattle spermatozoa across days 1-3 of storage treatment days, statistical analysis was conducted using two-way ANOVA with a 5% significance level.

# 3 Results and discussions

## 3.1 Results

Fresh Bali cattle semen was evaluated before proceeding with the dilution process. The evaluation of fresh semen was conducted both macroscopically and microscopically. The results indicated that the fresh semen fell into the good category and met the requirements, with a motility value of ≥ 70% and abnormalities ≤ 20% [10]. The detailed results of the evaluation of fresh Bali cattle semen are presented in Table 1.

**Table 1.** Evaluation Results of Fresh Semen Quality of Bali Cattle

| Parameter | Average ± SD |
|---|---|
| Color | Milky white |
| pH | 6.7 ± 0.1 |
| Consistency | Moderate |
| Volume (ml) | 7 ± 1.7 |
| Concentration (million/ml) | 1.117 ± 570.6 |
| Individual Motility (%) | 72.3 ± 4.0 |
| Abnormality (%) | 10.7 ± 2.0 |
| Viability (%) | 90.2 ± 1.6 |
| Membrane Integrity (%) | 73.7 ± 3.9 |

The results of the fresh semen evaluation showed that the average value of fresh semen, which was 7±1.7 ml, was higher compared to the observation results of Fazrien *et al.* [11], which ranged from 5.5 to 6.9 ml. The volume comparison is influenced by several factors, including the male's age, condition, and the environmental conditions during semen production [12]. The fresh Bali cattle semen obtained was milky white, indicating it was in normal condition. The color of the semen can be used as an indicator of semen quality. Clear semen indicates a low sperm concentration, yellow semen indicates urinary contamination, and red semen indicates blood contamination [13].

The pH value of fresh semen was 6.7, which falls within the normal range, as according to Manehat *et al.* [14], the normal pH of cattle semen is between 6.0 and 7.8. The metabolic mechanisms of spermatozoa cells influence the pH value of semen in male cattle in breaking down the fructose energy source, with high levels of lactic acid resulting in a more acidic semen pH [11]. The consistency of fresh Bali cattle semen was categorized as moderate, with a concentration of 1117 million per ml. Semen consistency is closely related to sperm concentration, meaning that the thicker the semen, the higher the sperm concentration. Male cattle may have varying consistency and concentration, influenced by age, scrotal circumference, and feed quality [15].

The observation of motility and spermatozoa abnormalities was conducted objectively with Computer-Assisted Semen Analysis (CASA). The progressive motility of Bali cattle spermatozoa was 72.3%, with an abnormality rate of 10.7%. These values indicate that the semen is of good quality and meets the requirements: progressive sperm motility ≥

70% and abnormalities ≤ 20% [10]. The progressive motility of spermatozoa in this study was lower compared to the research by Sawitri *et al.* [16], which reported a value of 76%. The age of the male cattle used influences the difference in motility values. In this study, the Bali cattle used were between 8 and 11 years old, older than the 6-7-year-old Bali cattle used in the Sawitri *et al.* [16] study. This is consistent with the statement by Pardede *et al.* [17] that as cattle age increases, progressive motility tends to decrease.

Spermatozoa viability was observed using the eosin-nigrosin staining method, while membrane integrity was assessed using the Hypoosmotic Swelling Test (HOST). The viability percentage of Bali cattle spermatozoa was 90.2%, which falls into the good category, as it is ≥ 70% [3] —the observation of spermatozoa membrane integrity aimed to assess the integrity of the sperm cell plasma membrane. The percentage of spermatozoa membrane integrity was 73.7%, higher than the result observed by Bahmid [16], which was 63.49%. The difference in membrane integrity values is influenced by factors such as feed quality, the age of the male cattle, and environmental conditions [19]. This study aims to compare various types of antioxidants that can maintain the quality of Bali cattle spermatozoa during storage at 5°C. The quality parameters used include motility, viability, and spermatozoa membrane integrity. The results of the spermatozoa motility observations, stored at 5°C up to the third day, were shown in Table 2.

**Table 2.** Motility of Bali Cattle Spermatozoa Stored at 5°C up to the 3rd Day

| Treatment | Motility Percentage on Day- (%) ± SD | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| P1 (CEP = Control) | 58.3 ± 2.4a | 56.5 ± 0.5 a | 43.7 ± 5.5 a | 27.0 ± 13.8 a |
| P2 (CEP + 2 mM alpha-tocopherol) | 55.9 ± 0.9 a | 59.6 ± 0.9 ab | 48.2 ± 4.7 ab | 35.7 ± 4.1 ab |
| P3 (CEP + 0.75 mM glutation) | 60.1 ± 0.3 a | 60.5 ± 0.3 ab | 50.3 ± 2.3 ab | 37.6 ± 4.6 ab |
| P4 (CEP + 2 mM alpha-tocopherol + 0.75 mM glutathione) | 61.4 ± 5.2 a | 63.4 ± 6.5 b | 52.1 ± 2.0 b | 42.4 ± 1.6 b |
| P5 (Tris Egg Yolk) | 57.4 ±3.0 a | 55.5 ±0.6 a | 44.6 ± 4.1 ab | 36.7 ± 5.7 ab |

Table 2 showed that the average percentage of spermatozoa motility stored at 5°C did not differ significantly on day 0 across the five treatments. However, the motility percentage showed significant differences on days 1-3 of storage. Based on the results of this study, the combination treatment of 2 mM alpha-tocopherol + 0.75 mM glutathione resulted in the highest motility percentage compared to the other treatments, with values of 61.4±5.2% on day 0, 63.4±6.5% on day 1, 52.1±2.0% on day 2, and 42.4±1.6% on day 3. The lowest percentages were obtained from 0.2 mM alpha-tocopherol on day 0 (55.9±0.9%), tris egg yolk extender on day 1 (55.5±0.6%), and CEP extender without antioxidants on days 2 and 3, with percentages of 43.7±5.5% and 27±13.8%, respectively.

The results of spermatozoa viability observations stored at 5°C up to the third day are shown in Table 3.

**Table 3.** Viability of Bali Cattle Spermatozoa Stored at 5°C up to the 3rd Day

| Treatment | Viability Percentage on Day- (%) ± SD | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| P1 (CEP = Control) | 88.2 ± 2.6 a | 83.7 ± 4.9 a | 77.9 ± 1.4 ab | 75.3 ± 0.8 ab |
| P2 (CEP + 2 mM alpha-tocopherol) | 90.7 ± 3.6 a | 84.4 ± 2.6 a | 82.1 ± 2.3 b | 76.9 ± 2.3 b |
| P3 (CEP + 0.75 mM + 0.2 Mm alpha-tocopherol) | 87.5 ± 2.0 a | 80.1 ± 5.8 a | 75.3 ± 1.5 a | 73.4 ± 3.5 ab |
| P4 (CEP + 2 mM alpha-tocopherol + 0.75 Mm glutathione) | 88.2 ± 6.6 a | 83.1 ± 5.5 a | 78.0 ± 5.1 ab | 73.1 ± 1.3 ab |
| P5 (Tris Egg Yolk) | 87.7 ± 6.8 a | 80.6 ± 3.1 a | 76.7 ± 4.7 ab | 72.1 ± 2.3 a |

Table 3 showed that the average percentage of spermatozoa viability stored at 5°C did not differ significantly on days 0 and 1 across the five treatments. However, the viability percentage of spermatozoa showed significant differences on days 2-3 of storage. Based on the results of this study, the treatment that minimized spermatozoa cell death up to day three of storage was the addition of 2 mM alpha-tocopherol in the CEP extender. The viability percentages of spermatozoa with the addition of 2 mM alpha-tocopherol were 90.7±3.6% on day 0, 84.4±2.6% on day 1, 82.1±2.3% on day 2, and 76.9±2.3% on day 3. The lowest viability percentages on days 0, 1, and 2 were obtained from adding 0.75 mM glutathione, with values of 87.5±2.0%, 80.1±5.8%, and 75.3±1.5%, respectively. On day 3, the lowest viability percentage was obtained with the Tris egg yolk extender without antioxidants, at 72.1±2.3%. The results of spermatozoa membrane integrity observations stored at 5°C up to the third day are shown in Table 4.

**Table 4.** Membrane Integrity of Bali Cattle Spermatozoa Stored at 5°C up to the 3rd Day

| Treatment | Percentage of Spermatozoa Membrane Integrity on Day- (%) ± SD | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| P1 (CEP = Control) | 86.6 ± 1.1c | 77.2 ± 1.8b | 65.1 ± 3.9bc | 57.7 ± 2.1 ab |
| P2 (CEP + 2 mM alpha-tocopherol) | 89.2 ± 1.3d | 80.8 ± 2.4c | 69.1 ± 1.8c | 59.4 ± 2.3 b |
| P3 (CEP + 0.75 mM + 0.2 Mm alpha-tocopherol) | 85.6 ± 1.8bc | 77.9 ± 0.9b | 64.0 ± 1.8 abc | 56.5 ± 1.7ab |
| P4 (CEP + 2 mM alpha-tocopherol + 0.75 Mm glutathione) | 84.0 ± 1.5ab | 76.2 ± 1.7b | 61.8 ± 2.3ab | 56.2 ± 2.6 ab |
| P5 (Tris Egg Yolk) | 81.6 ± 1.1a | 72.5 ± 0.8a | 59.6 ± 3.6a | 55.3 ± 3.2a |

Table 4 showed that the average percentage of spermatozoa membrane integrity stored at 5°C differed significantly from day 0 to day 3. Based on the results of this study, the treatment that maintained the integrity of spermatozoa membranes up to the third day of storage was the addition of 2 mM alpha-tocopherol in the CEP extender. The membrane integrity percentages of spermatozoa with the addition of 2 mM alpha-tocopherol were 89.2±1.3% on day 0, 80.8±2.4% on day 1, 69.1±1.8% on day 2, and

59.4±2.3% on day 3. The lowest spermatozoa membrane integrity percentages from day 0 to day 3 were obtained using the Tris egg yolk extender, with values of 81.6±1.1%, 72.51±0.8%, 59.63±3.6%, and 55.3±3.2%, respectively.

### 3.2 Discussion

This study aimed to store Bali cattle semen at 4–5°C for three days, during which sperm quality—motility, viability, and membrane integrity—declined. Low-temperature semen storage cannot counteract free radicals, leading to lipid peroxidation, which reduces motility [20]. Free radicals are by-products of slow spermatozoa metabolism during low-temperature storage. Ducha *et al.* [4] explained that adding antioxidants to extenders minimizes lipid peroxidation.

In observing sperm motility, the combination of 2 mM alpha-tocopherol and 0.75 mM glutathione demonstrated better results than other treatments. To date, the addition of combined antioxidants and glutathione in semen extenders has not been studied. The findings align with Reyes *et al.* [21], who combined the antioxidant alpha-tocopherol (4 mg/ml) with another antioxidant, quercetin (25 μM), achieving sustained motility in frozen goat sperm.

Storing sperm at low temperatures for an extended period reduces sperm viability as metabolism continues slower. Martemucci *et al.* [22] noted that metabolism generates free radicals by product, triggering lipid peroxidation in membranes. Free radicals are highly reactive, and if unchecked, they initiate continuous chain reactions, ultimately damaging the entire sperm plasma membrane [23].

This study also observed membrane integrity, which correlates with sperm viability. Gungor *et al.* [24] stated that sperm viability and membrane integrity are critical factors in determining proper sperm functionality, including the ability to reach and fertilize the egg. Therefore, it can be concluded that sperm viability and membrane integrity are closely related to the condition of the spermatozoa plasma membrane.

Observations of motility showed different results compared to sperm viability and membrane integrity. The best percentages were obtained for viability and membrane integrity by adding two mM alpha-tocopherol to the CEP extender. However, all three parameters demonstrated a consistent finding: the CEP extender supplemented with alpha-tocopherol antioxidants effectively maintained sperm quality.

Alpha-tocopherol, a vitamin E antioxidant, inhibits lipid peroxidation in sperm membranes [25]. Sperm plasma membranes are composed of unsaturated fatty acid phospholipids, highly susceptible to free radicals and lipid peroxidation [26]. Adding alpha-tocopherol to the CEP extender prevents lipid peroxidation, preserving sperm plasma membrane integrity.

Alpha-tocopherol reacts directly with free radicals that trigger lipid peroxidation. According to Ducha *et al.* [4], alpha-tocopherol neutralizes free radicals by donating a hydrogen atom from its OH group to stabilize the radical molecule. This reaction produces a more stable tocopherol radical that does not harm sperm cells.

Another antioxidant used in this study was glutathione. Adding 0.75 mM glutathione yielded the best results for sperm motility. This finding aligns with research by Zou *et al.* [27], which found that adding glutathione to semen extenders preserved the motility of

frozen sperm. The results suggest that glutathione has a more significant influence on motility than sperm viability and membrane integrity.

Glutathione is believed to counteract free radicals that compromise membrane integrity and affect mitochondria, which are crucial for sperm motility. Glutathione neutralizes free radicals by donating electrons [28]. This mechanism is supported by Aaseth *et al.* [29], who stated that glutathione contains cysteine (SH) molecules that donate electrons to odd-electron radical molecules, thus neutralizing them.

Based on data from observations of motility, viability, and membrane integrity, Bali cattle sperm can be stored in CEP diluent. However, there were differences in the quality profile after storage for three days. The motility profile appeared highest in CEP diluent with the addition of alpha-tocopherol + glutathione. This differed from the membrane viability and integrity profile, which is the highest percentage in CEP diluent with the addition of alpha-tocopherol.

## 4 Conclusions

Bali cattle sperm can be stored at a low temperature of 4–5°C in a CEP extender by adding different antioxidants. The best sperm motility and viability were observed in the CEP extender supplemented with alpha-tocopherol. In contrast, the best membrane integrity was found in the CEP extender with the addition of alpha-tocopherol and glutathione. Based on the study results, Bali cattle sperm stored at a low temperature of 4–5°C meets the requirements for future artificial insemination.

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