Cryopreservation of Bali Bull Semen in CEP Diluent with the Addition of Various Antioxidants

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Abstract. Cryopreservation of semen was a crucial technology in livestock breeding; however, it compromised sperm quality due to oxidative stress, lipid peroxidation, and structural damage. This study aimed to determine the effect of adding α-tocopherol and glutathione antioxidants in Caudal Epididymal Plasma (CEP) diluent on the quality of frozen Bali bull semen. Four groups of CEP semen diluents were prepared with different antioxidant supplementation: P1 (CEP), P2 (CEP + 2 mM α-tocopherol), P3 (CEP + 0.75 mM glutathione), and P4 (CEP + 2 mM α-tocopherol + 0.75 mM glutathione), while Tris Egg Yolk extender was used as a comparator (P5). Semen collected from two Bali bulls was evaluated before and after the freezing and thawing processes using parameters such as motility, viability, membrane integrity, lipid peroxidation (MDA), and DNA integrity. The results showed that the P2 group supplemented with 2 mM α-tocopherol had the highest post-thawed motility, viability, and membrane integrity compared to other groups ($P<0.05$). The addition of α-tocopherol and glutathione proved to reduce MDA concentrations and improve the quality of frozen semen. This study concluded that α-tocopherol and glutathione improved the quality of Bali bull semen after cryopreservation, with α-tocopherol showing more significant results.
Keywords: Antioxidants, bali bull, caudal epididymal plasma (CEP), lipid peroxidation, semen cryopreservation

1 Introduction

The application of reproductive technology, specifically semen cryopreservation, in the livestock industry represents a modern genetic strategy in breeding programs, aiming to preserve germplasm [1]. Cryopreservation of local livestock semen has also been widely practiced to preserve its viability. However, cryopreserved semen also negatively impacts spermatozoa cells, such as decreased motility and viability, damage to cell mitochondrial structure and function, DNA damage, and alteration of acrosome structure [2]. The process

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of freezing and thawing semen disrupts the biophysical and biochemical conditions of spermatozoa, as it undergoes cold shock, ice crystal formation, and oxidative stress [3].

The plasma membrane of mammalian spermatozoa, including that of cattle, is composed of phospholipids, glycolipids, and sterols [4]. The high lipid composition in the plasma membrane of spermatozoa makes it highly susceptible to lipid peroxidation during cryopreservation, which triggers the production of reactive oxygen species (ROS). Excessive ROS levels contribute to oxidative stress, lipid peroxidation, protein and cell damage [5], leading to a decline in the quality of spermatozoa cells. Efforts to maintain the quality of spermatozoa include adding semen diluents supplemented with antioxidants before the freezing process. Semen diluents are made from chemicals containing cryoprotectants, fatty acids, sugars, amino acids, and membrane stabilizers [1]. Thus, research on the use of semen diluents from various materials containing antioxidants needs to be carried out, especially for the processing of Bali bull semen as an indigenous Indonesian cattle.

2 Materials and methods

2.1 Caudal Epididymal Plasma (CEP) extender preparation

The preparation of CEP diluent referred to Ducha (2018), with the composition of the diluent in one liter containing 15 mmol NaCl; 7.0 mmol KCl; 3.0 mmol CaCl2(H2O)2; 3.0 mmol NaHCO3; 3.0 mmol MgCl2 (H2O)6; 11.9 mmol NaHCO3; 8.0 mmol NaH2PO4; 20.0 mmol KH2PO4; 55 mmol fructose; 1.0 g sorbitol; 2.0 g BSA; 133.7 mmol Tris; 1000 IUI penicillin; 1 g streptomycin; 42.6 mmol citric acid dam 20% egg yolk. The CEP diluent was divided into four parts, namely P1 (CEP), P2 (CEP + 2 mM α-tochoperol), P3 (CEP + 0.75 mM glutathione), dan P4 (CEP + 2 mM α-tochoperol + 0.75 mM glutathione).

2.2 Tris egg yolk extender preparation

Tris Egg Yolk extender (TEY) in this study was used as a comparison (P5) and was produced by the Singosari National Artificial Insemination Center, Indonesia, with a composition of 1.6% Tris aminomethane, 0.9% citric acid, 1.4% lactose, 2.5% raffinose, 20% egg yolk, 100.00 IU/100ml penicillin, 0.1 g/100 ml streptomycin, 13% glycerin, and 80% distilled water.

2.3 Semen collection and semen evaluation

Collection was carried out twice a week from two Bali bulls aged 8 and 13 years, which were well-maintained by the Artificial Insemination Centre, Singosari, Malang. These bulls had a regular weekly semen collection schedule, periodic health checks, and feedings according to the standards set by the institute. Semen collection was performed using the artificial vagina method in the morning, with at least two ejaculations collected from each bull. Fresh semen from the bulls had to meet the requirements set by the Indonesian National Standard, namely progressive sperm motility ≥ 70% and sperm abnormalities ≤ 20%. Macroscopic evaluation of fresh semen included color, pH, volume, odor, and consistency. Microscopic evaluation of fresh semen included motility and abnormalities using Computer-Assisted Sperm Analysis (CASA), concentration using an SDM 6 Minitube photometer, viability using the eosin-nigrosin staining method, and membrane integrity using the hypo-osmotic swelling test (HOST).

2.4 Semen processing

The dilution of fresh semen was carried out in three stages. The first dilution (A1) was performed at 37°C with a dilution ratio of 1:1 for diluent and semen. The semen was then cooled to 4°C in a refrigerator. The second dilution stage (A2) was carried out at 4°C and stored in the refrigerator for 18-24 hours. The third dilution stage was performed by adding diluent B (CEP/TKT diluent + 13% glycerol) equal to half of the total volume. The diluted semen was then equilibrated at 4-5°C for approximately 1 hour and evaluated before freezing. The liquid semen was placed into 0.25 ml straws using a Filling and Sealing machine. The pre-freezing process was carried out using a DigitCool machine, followed by freezing by dipping the straws containing the liquid semen into liquid nitrogen at -196°C for a few seconds. Post-thawing evaluation of frozen semen was conducted by thawing it in a 37 °C water bath for 30 seconds.

2.5 Sperm evaluation

2.5.1 Motility

The evaluation of sperm motility was conducted objectively using CASA at 37°C. A semen sample (3-5 µl) was placed on a microscope slide and covered with a cover glass.

2.5.2 Viability

The viability of spermatozoa was tested using eosin-nigrosin staining. A semen sample was placed on the edge of a microscope slide, stained (1:1), and then homogenized. A thin smear was then prepared and observed using an Olympus light microscope at 400x magnification until approximately 200 spermatozoa cells were counted. The percentage of spermatozoa viability was calculated based on the following formula:

$$\%\text{Viability} = \frac{\text{total of live sperm}}{\text{total sperm (live and dead)}} \text{ x } 100\% \quad (1)$$

2.5.3 Membrane integrity

The integrity of the spermatozoa membrane was tested using the Hypoosmotic Swelling Test (HOST) solution. A semen sample and HOST solution (1:10) were homogenized and then incubated in a 37 °C water bath for 30 minutes. A thin smear was then prepared and observed using an Olympus microscope at 400x magnification until approximately 200 spermatozoa cells were counted. The percentage of spermatozoa membrane integrity was calculated based on the following formula:

$$\%\text{Membran Integrity} = \frac{\text{total of coiled tail sperm}}{\text{total sperm}} \text{ x } 100 \quad (2)$$

2.5.4 Lipid peroxidation

Lipid peroxidation was determined using the malondialdehyde (MDA) assay and conducted on semen samples before and after freezing. This assay was performed using the QuantiChromTM TBARS Assay Kit with the colorimetric method. The procedure began by establishing the standards used. The samples were then centrifuged at 1000 rpm for 15 minutes to separate the diluent and sperm cells (pellet). Next, 200 µL of 10% TCA was added

and incubated on ice for 5 minutes. The mixture of sperm cells and 10% TCA was centrifuged at 14000 rpm for 5 minutes, resulting in the formation of a supernatant. The supernatant was collected and placed into a new tube, followed by the addition of 200 µL TBA reagent. The samples were incubated at 100°C for 60 minutes. The samples were then cooled and briefly vortexed. The results were placed into a cuvette and read on a spectrophotometer at a wavelength of 535 nm.

2.5.5 DNA integrity

The DNA integrity test of spermatozoa began with DNA extraction to obtain pure spermatozoa DNA. The DNA extraction of spermatozoa was carried out using a DNA extraction kit from Jena Bioscience. Next, the concentration and purity of spermatozoa DNA were measured using a Nano Drop spectrophotometer and visualized with a 0.8% agarose gel.

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2.6 Statistical analysis

The average data of sperm motility, viability, and membrane integrity were tested for normality using the Shapiro-Wilk test ($P>0.05$), then analyzed using one-way ANOVA ($P<0.05$) to determine the effect of treatments, and the Duncan test to observe the differences among treatments.

3 Results and discussion

3.1 Fresh semen quality

Colour, pH, consistency, volume, concentration, motility, viability, and membrane integrity of sperm all indicate that Bali bull fresh semen is of high quality (Table 1). The quality of fresh Bali bull semen shows good quality characterized by milky white color and thick consistency [6]. The higher the semen volume, the greater the male's potential to produce more frozen semen. Volume and concentration of semen serve as the foundation for figuring out how much semen diluent should be added and, eventually, how much frozen semen should be generated. Based on the Indonesian National Standard (2024) with number: RSNI3 4869-1:2024 on frozen semen, the quality of fresh semen of Bali bull in this study is suitable for the freezing process because it has motility >70%, and abnormality <20%.

Table 1. Evaluation of the quality of fresh Bali bull semen.

Parameter	Mean ± Standard Deviation
Color	Milky
pH	6.70
Consistency	Thick
Volume (ml)	7.4±1.2
Concentration (million/ml)	1,457±66.5
Individual Motility (%)	73.2±2.1
Abnormality (%)	8.3±5.7
Viability (%)	87.4±0.4
Membrane integrity (%)	74.7±4.5

3.2 Frozen semen quality

The data showed that there was a decline in the quality of spermatozoa from fresh semen to frozen semen due to the dilution and freezing processes. [7] Previously stated that the cryopreservation of spermatozoa could decrease their quality. To minimize the decline in spermatozoa quality during cryopreservation, we added semen diluent media, namely Caudal Epididymal Plasma (CEP). The CEP media used was a modification [8] with different types of antibiotics, egg yolk concentration, and production techniques. In this study, we found that adding CEP diluent media before cryopreservation was highly suitable for the storage of bull spermatozoa because the press was made to match the conditions of the bull's epididymis. Additionally, the composition of CEP provided energy sources (fructose and sorbitol), ionic sources (Na, K, Ca, Cl, Mg, P), stabilized pH (tris and citric acid), antibiotics (streptomycin and penicillin), and extracellular cryoprotectants (20% egg yolk). Only CEP media was certainly not effective enough to minimize the appearance of ROS during the dilution and freezing processes; therefore, it was necessary to add extracellular antioxidants. Semen naturally contains antioxidants, but the oxidative stress triggers the excessive production of ROS, which is detrimental to spermatozoa.

Cryopreservation of semen went through several stages before being frozen at -196°C, namely dilution and cooling. This process could reduce the stock of enzymatic antioxidants, thus reducing the performance of endogenous semen antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) [1]. Additionally, the plasma membrane of spermatozoa is composed of Polyunsaturated Fatty Acids (PUFA), making it susceptible to oxidative damage (9) (Evans et al., 2021). ROS targets PUFA, causing lipid peroxidation and the formation of new radicals (lipid peroxidation chain reaction) [10].

As shown in Table 2, the P2 grup consistenly demonstrated the best results across several parameters. The highest equilibrated sperm motility was found in the P2 group (61.20±1.10%), while the lowest in the P3 group (52.8±1.9%). Similarly, in the post-thawed observation, motility was highest in the P2 group (46.6±2.1%) and lowest in the P5 group (36.7±2.9%), with the P2 group showed a significant difference compared to the other four groups ($P<0.05$). For sperm viability, the highest equilibrated value was observed in the P2 group ((81.5±2.1%), which has signifcantly different from the other groups ($P<0.05$), while he lowest was in the P5 group (73.9±2.0%). After thawing, the P2 group also showed the highest viability (71.3±0.5%), not significantly different from P4, and the lowest value was in the P1 group (73.9±2.0%). Regarding membrane integrity, the highest equilibrated value was recorded in the P4 group (72.8±1.3%) and the lowest in the P3 group (65.8 ±1.4%). However, in the post-thawed observation, the P2 group achieved the highest membrane

integrity (63.2±2.9%), significantly different from the other four groups ($P<0.05$), while the lowest was observed in the P3 group (55.3±2.7%).

In this study, the types of antioxidants used were α-tocopherol and glutathione, both of which fall under the category of vitamin E. The CEP group supplemented with two mM α-tocopherol was able to maintain the motility and viability of spermatozoa during equilibrated and post-thawed stages compared to other groups ($P<0.05$) (Table 2). This study was similar to [11], which found that two mM α-tocopherol in the semen diluent could maintain the motility and viability of spermatozoa post-freezing. Sperm motility and viability were positively correlated. The percentage of spermatozoa viability was greater than motility because not all live spermatozoa move progressively. Meanwhile, membrane integrity showed that α-tocopherol alone was able to maintain the integrity of spermatozoa membranes, whether combined with 0.75 mM glutathione or not.

The addition of α-tocopherol in CEP was believed to act as an antioxidant, thus able to suppress lipid peroxidation and reduce the presence of ROS, which showed that α-tocopherol in CEP diluent could also maintain the quality of spermatozoa stored at 4-5°C. α-tocopherol is a type of vitamin E that can reduce membrane damage due to lipid peroxidation by inhibiting excessive ROS production. This antioxidant is also one of the antioxidant compounds often found in plasma membranes and seminal plasma.

Table 2. Effect of antioxidants supplementation on motility, viability, and membrane integrity of Bali bull spermatozoa during equilibration and post-thaw stages.

Variabel	**Evaluation stage**	**Antioxidants supplementation**				
		P1	**P2**	**P3**	**P4**	**P5**
Motilitas (%)	Equilibrated	$57.8±1.9^{ab}$	$61.2±1.1^{a}$	$52.8±1.9^{c}$	$57.8±1.9^{ab}$	$57.2±2.6^{b}$
	Post-thawed	$40.6±1.9^{b}$	$46.6±2.1^{a}$	$40.4±0.4^{b}$	$40.0±4.4^{b}$	$36.7±2.9^{b}$
Viability (%)	Equilibrated	$76.6±2.2^{b}$	$81.5±2.1^{a}$	$76.5±1.5^{b}$	$76.5±1.7^{b}$	$73.9±2.0^{b}$
	Post-thawed	$63.5±4.4^{b}$	$71.3±0.5^{a}$	$64.7±1.0^{b}$	$71.0±3.5^{a}$	$64.0±1.4^{b}$
Integrity membrane (%)	Equilibrated	$70.0±0.7^{ab}$	$70.9±5.6^{ab}$	$65.8±1.4^{b}$	$72.8±1.3^{a}$	$66.3±0.8^{b}$
	Post-thawed	$56.0±1.7^{b}$	$63.2±2.9^{a}$	$55.3±2.7^{b}$	$56.8±2.4^{b}$	$55.7±1.4^{b}$

Values are presented as mean ± standard deviation (SD).
Different superscript letters within the same row indicate significant differences ($P<0.05$).
P1 (CEP); P2 (CEP + 2 mM α-tochoperol); P3 (CEP + 0,75 mM glutathione); P4 (CEP + 2 mM α-tochoperol + 0,75 mM glutathione); P5 (Tris egg yolk)

The mechanism by which α-tocopherol minimizes ROS production is by reducing H_2O_2 during cryopreservation [12]. This antioxidant also played an essential role in reducing the impact of sperm plasma membrane damage by decreasing ROS. The plasma membrane acted as a protective barrier for spermatozoa from the external cellular environment [13]. The conditions of spermatozoa motility, viability, and membrane integrity were related to the integrity of the plasma membrane so that membrane damage would decrease spermatozoa quality.

Glutathione is a tripeptide compound formed from the condensation of glutamic acid, cysteine, and glycine [14]. Cysteine in glutathione acts as an electron donor to ROS [15], stabilizing the ROS molecules. Additionally, glutathione also plays a vital role in protecting spermatozoa from oxidative stress through detoxification mechanisms. Similar to the antioxidant α-tocopherol, glutathione also removes H_2O_2, which is responsible for the lipid peroxidation process. The addition of glutathione also had a positive impact on reducing MDA, although its effect was not as strong as that of α-tocopherol.

Many studies have mentioned that cells experience oxidative stress during cryopreservation due to excessive ROS production. The presence of ROS was closely related to lipid peroxidation in the spermatozoa cell membrane. To detect the presence of ROS, the concentration of malondialdehyde (MDA) in spermatozoa cells post-thawing can be measured, as MDA is a substance produced from lipid peroxidation.

3.3 DNA Profile of Bali Bull Spermatozoa Before and After Freezing

The results of agarose gel electrophoresis are presented in Figure 1. Electrophoresis was performed to determine the number of DNA base pairs present in Bali bull spermatozoa subjected to different treatments. The results showed variations in the thickness and intensity of the protein bands formed.

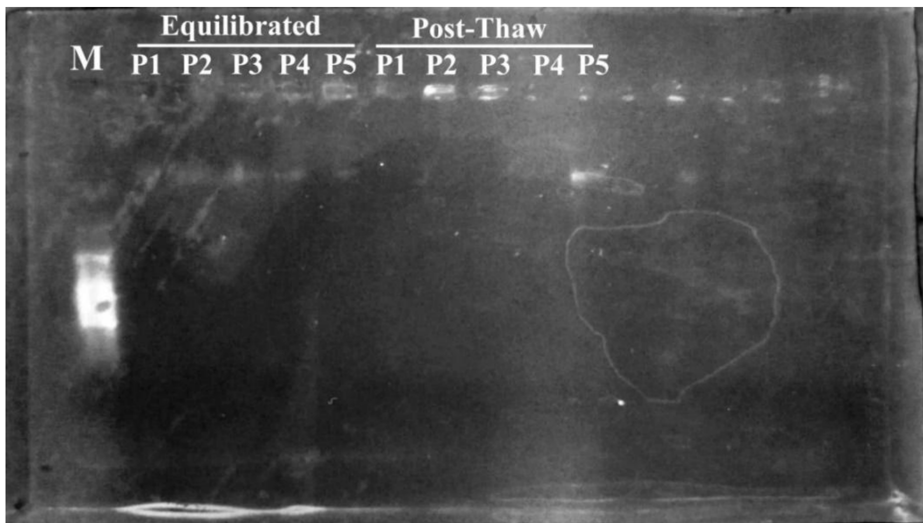

Figure 1. Agarose gel electrophoresis of Bali bull sperm equilibrated and post-thaw.

The electrophoresis results of spermatozoa before freezing (BF) and after freezing (AF) are shown. Code M represents the protein marker; P1 (CEP), P2 (CEP + 2 mM α-tocopherol), P3 (CEP + 0.75 mM glutathione), P4 (CEP + 2 mM α-tocopherol + 0.75 mM glutathione), and P5 (CEP + Tris aminomethane).

The electrophoresis results of spermatozoa before freezing (BF) showed that no protein bands were observed in treatments P1 and P2. Thin protein bands were observed in treatments P3 and P4, while treatment P5 showed a thicker protein band compared to the other treatments. In contrast, the electrophoresis results of spermatozoa after freezing (AF) showed that treatment P2 produced a thicker band than the other four treatments. Treatment P1 showed a thin band, P3 showed a thick band, whereas no protein bands were observed in treatments P4 and P5. The thickness of the protein bands indicates the protein content of the samples. The thicker the protein band formed, the higher the protein content. It can be concluded that spermatozoa samples preserved in the CEP extender supplemented with 2 mM α-tocopherol exhibited the best DNA protein content compared to the other treatments.

4 Conclusion

This study concluded that the supplementation of α-tocopherol and glutathione in CEP diluent can improve the quality of Bali bull semen post-cryopreservation, with α-tocopherol showing more significant results. We would like to extend our deepest gratitude to the State University of Surabaya, Surabaya, East Java, Indonesia, for all the support provided, enabling this product innovation development research project to be carried out successfully. We are also very grateful to the Singosari National Artificial Insemination Center, Malang, East Java, Indonesia, for being a valuable research partner and providing the venue for conducting this research.

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