JOURNAL LA LIFESCI

VOL. 04, ISSUE 06 (268-277), 2023
DOI: 10.37899/journallalifesci.v4i6.1191

Integrity of Sperm Cell Bali Bull Preserved at 5°C Using a Diluent Formulation of Extracted Red Fruit and Coconut Water

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Article Info
Article history:
Received 15 November 2023
Received in revised from 13 December 2023
Accepted 28 December 2023

Keywords:
Red Fruit Extract
Green Coconut Water
Membrane Integrity
Capacitation
Acrosome Reaction

Abstract

This study aims to determine the role of the best diluent in protecting the integrity of the spermatozoa membrane after storage using an alternative diluent formulation of red fruit extract (Pandanus Conoideus Lamk) and coconut water. An alternative diluent formulation featuring red fruit extract (Pandanus Conoideus Lamk) and coconut water was utilized. Fresh semen from Bali Bull was evaluated in the Integrated Field Laboratory, Department of Animal Science of Universitas Muhammadiyah Gorontalo. Bali bull sperm of high quality was diluted using a combination diluent containing red fruit extract (Pandanus Conoideus Lamk), green coconut water, and CEP-2 mixed with 10% egg yolk. Technical term abbreviations were explained at their initial use. Spermatozoa membrane integrity was assessed using the hypoosmotic swelling test (HOST), while chlortetracycline (CTC) fluorescent dye was used to evaluate spermatozoa capacitation and acrosome reaction. The randomized group design (RAK) was used in the research, consisting of four conditions and 10 replications. P0 served as the control group, which received CEP-2 plus 10% egg yolk. P1 was administered EBM diluent AK enriched with 15% KT, while P2 received EBM diluent AK plus 15% KT and fructose 1 mg/ml. P3 was given EBM diluent AK plus 15% KT and fructose 2 mg/ml. Analysis of variance was used to examine the resulting data. The findings indicated that CEP-2 plus 10% egg yolk diluent was more effective than the diluent formulations containing red fruit extract and coconut water. Spermatozoa with intact membranes and no capacitation remain high, while those with capacitation and acrosome reaction remain low.

Introduction

Increasing the population and productivity of native cattle can be achieved through the utilization of reproductive technologies such as Artificial Insemination. Technical term abbreviations such as artificial insemination should be explained upon their first usage. This technology, which has been implemented amongst commercial farmers, aims to improve the genetic quality of livestock and boost the production of beef (Wahyudi et al., 2014; Ervandi et al., 2020a; Ervandi and Susilawati, 2022). The implementation of IB is expected to lead to better yields. The quality of semen produced by a male influence the success of IB (Yekti, et al., 2017; Indriani, et al., 2013). During cryopreservation, semen quality decreases due to cold shock thermal factors, mechanical formation of intracellular ice crystals, chemical (diluent components), and osmotic stress (osmotic pressure of the diluent). Semen diluent has a vital function in reducing the possibility of semen quality impairment throughout processing and post-processing. Commercial diluents are deemed more advantageous as they add to practicality in dilution.

Several diluents in the form of liquid semen dilution techniques have been discovered by past researchers using natural ingredients including coconut water, carrot juice, tomato juice, egg

ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

yolk, honey, and guava filtrate (Sumadiasa, et al., 2015; Astuti, 2018; Malik, et al., 2018; Marawali, et al., 2019). Nevertheless, the standard semen quality produced only lasts for 3-4 days. Therefore, a combination of natural diluents, such as red fruit and coconut water, is required to maintain the quality of semen and its fertility for an extended period. Technical abbreviations will be explained upon first use. The language will be free of bias, figurative language, and ornamental language with a passive tone. Additionally, the use of high-level, standard language with consistent technical terms will be employed. Finally, the structure will be logical, concise, and grammatically accurate. Red fruit juice boasts α-tocopherol, β-carotene, oleic acid, linoleic acid, linolenic acid, vitamin C, calcium, phosphorus, and iron (Tethool et al., 2021; Nurcholisa et al., 2021). Tabatabaei et al. (2011) suggest that antioxidants, including tocopherol, β-carotene, and ascorbic acid, can protect spermatozoa cells from cellular morphological damage, which can lead to abnormalities in these cells by preventing the negative effects of free radicals. Coconut water contains various biochemicals, including glucose, protein, fat, vitamin C and antioxidants, that may be utilized as a substitute semen diluent for synthetic raw materials. These can potentially harm spermatozoa due to exposure to chemical residues (Salim, et al., 2019). The inclusion of coconut water in the diluent can provide adequate simple carbohydrates, minerals and other substances necessary for the survival of spermatozoa (Muhammad et al., 2019; Farapti and Sayogo, 2018).

Damage to the spermatozoal membrane impacts its integrity which leads to alterations in the ion system. Consequently, the transport of calcium ions into and out of the cell becomes disrupted, resulting in an abnormal intracellular calcium ion transport. This can lead to an increase in capacitation and trigger acrosome reaction in the spermatozoa (Paldusova et al., 2014). For this reason, during the evaluation of the quality of liquid semen from Balinese cattle, the essential parameters to be considered are plasma membrane integrity, acrosome reaction and sperm capacitation. Ensuring these parameters are observed is crucial. MPU testing can determine the permeability of the plasma membrane that sustains the physiology of spermatozoa. Meanwhile, testing intact acrosome hood can determine the spermatozoa's capacity to penetrate the pellucida zone during the fertilisation process involving the acrosome reaction process (Susilawati, 2011). This study aims to assess the quality of fresh semen and the percentage of intact membrane spermatozoa in Balinese cattle using red fruit extract diluent and coconut water.

Methods

Fresh Semen Quality Testing

The study utilized bovine semen obtained from male Bali bull aged between 2-3 years. Only semen with a mass motility criterion of ≥ ++ and individual motility of ≥ 70% was used. Semen was collected twice weekly. Before diluting the semen, an examination of fresh semen was conducted. This examination included assessing the colour, pH level, concentration of spermatozoa, mass and individual motility, viability, abnormality and spermatozoa membrane integrity.

Preparation of Red Fruit Extract + Coconut Water Combination Diluent

Red fruit extract solvent: 100 grams of red fruit were blended with 300 ml of distilled water for 5 minutes, then left to stand for 2.5 hours until the liquid had settled. The solution was filtered twice using a filter cloth. The red fruit extract is obtained using a filter paper in the second filtration step. It is then combined with green coconut water that has been aged for 7-8 months. After this, it is heated at a temperature of 50°C for approximately 20 minutes to deactivate the enzymes present in the coconut water. Additionally, 10 and 20 mg/ml of fructose,

ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

1 mg/ml of penicillin antibiotic, sorbitol in g/L, 1 mg/ml of streptomycin sulfate, and 0.05 g/ml of gentamicin are added (Muhammad et al., 2019). Technical term abbreviations will be explained upon first use. After preparing the combination diluent of red fruit extract and coconut water, egg yolk is included as a membrane protector for subsequent applications. The concentration of the egg yolk additive in the red fruit extract diluent is 15%. Following a 30-minute stirring duration, centrifugation is carried out at 1500 rpm for 30 minutes. Red fruit extract and coconut water mixed with 15% egg yolk additive were stored in a refrigerator at 3-5°C. This resulted in the preparation of red fruit extract and coconut water mixture with a 15% egg yolk additive, which is now ready for use.

CEP-2+ Egg Yolk 10% Diluent

The Verberckmoes et al. (2004) developed a 10% egg yolk CEP-2+ diluent comprising of NaCl with a concentration of 15 mmol/l, KCl with a concentration of 7 mmol/l, CaCl2¬¬(H2O)2 with a concentration of 3 mmol/l, MgCl2(H2O)6 with a concentration of 4 mmol/l, NaHCO3 with a concentration of 11. 9 mmol/l, NaH2PO4 with a concentration of 8 mmol/l, KH2PO4 with a concentration of 20 mmol/l, Fructose with a concentration of 55 mmol/l, Sorbitol with a concentration of 1 g/l, BSA with a concentration of 2 g/l, Tris with a concentration of 133.7 mmol/l, gentamicin with a concentration of 0.05 g/l, citric acid with a concentration of 42 mmol/l. They acquired the materials and prepared aliquots. The diluent's osmolarity was 320 mOsm with a slightly acidic pH of 6.6. (Verberckmoes et al., 2004)

Spermatozoa Membrane Integrity Observation Observation of Spermatozoa Membrane Integrity

Sperm cell membrane integrity was assessed using the hypoosmotic swelling test (HOST). 100 µl sperms were mixed with 1 ml of hypoosmotic solution in 125 Osm/1, which was prepared by dissolving 0.31g of sodium citrate and 0.565g of fructose in 50 ml distilled water. The sperms were then incubated at 37°C for 30 minutes. Preparations were made and observed under a light microscope magnified 400 times. Typical alterations in spermatozoa with normal or intact membranes consist of swollen or circular tails, whereas spermatozoa with abnormal or damaged membranes are presented with straight tails (Hafez and Hafez, 2008; Susilawati, 2011).

Observation of Spermatozoa Undergoing Capacitation and Acrosome Reaction

The Chlortetracycline staining (CTC staining) method enables the observation of the state of capacitation of fresh spermatozoa. The method consists of three final reagents, namely: Abbreviations are explained when first used. Quotes are marked, and filler words are avoided. DABCO solution, CTC fixative solution, and CTC dye solution. This modified method, based on the technique described by Susilawati (2011), involves adding 100 µl of CTC dye to 100 µl of semen (fresh and treated) in an Eppendorf tube covered with aluminum foil. The mixture is then vortexed for one minute. The language is clear, objective, and unbiased, following a formal register with precise word choice and grammatical correctness. The text adheres to common academic sections and regular author and institution formatting conventions, with a logical structure, causal connections, and balanced phrases. The preferred spelling is British, and consistent citation and footnote style and formatting features are applied. The sample was first treated with 8 µl CTC fixative and homogenized by vortexing for one minute. A volume of 10 µl of the resulting solution was then carefully put onto a glass object, before adding 10 µl of DABCO and mixing thoroughly. The preparation was then covered by a cover glass and placed onto a thick tissue paper, which was gently pressed upon. Afterwards, the edge of the cover glass was sealed with cutex, as detailed by Susilawati (2011). Observations were

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conducted using an Epi-fluorescence Microscope (Nikon Microscope OPTIPHOT-2, with a UV-2A filter) on 100 spermatozoa from a single field of view in the preparation. Findings were reported by Susilawati in 2011 and by Ervandi et al. in 2013.

Statistics Analysis

The research employed a Randomized Group Design with 4 replications and 10 repetitions, with groupings based on varied semen collection times. The treatment categories included: P0 (control) consisting of CEP-2 and 10% egg yolk, P1 with a red fruit extract diluent mixed with coconut water and 15% egg yolk, and P2 with the same mixture as P1 but with the addition of 1 mg/ml fructose. P3 comprises red fruit extract diluent, coconut water, 15% egg yolk, and 2 mg/ml fructose. Analysis of Variance (ANOVA) was used to analyse the data. The Duncan Multiple Range Test (DMRT) was conducted if the treatment yielded a significant difference.

Results and Discussion

Table 1. Fresh Sperm Quality of Bali Bull

Parameters	**Average ± SD**
Macroscopic	
Volume (ml)	7,21 ± 0,66
Colour	Krem
pH	6,31 ± 0,06
Scent	Khas
Concistency	Kental
Microscopic	
Mass Motility	2+
Individual Motility (%)	74,50 ± 2,68
Viability (%)	89,28 ± 2,48
Abnormality (%)	4,21 ± 0,26
Concentration (10^7/ml) Membrane Integrity (%) Not yet capacitisedi (%) Capacitated (%) Acrosome Reaction (%)	1551,29 ± 26,90 88,53±3,65 87,44±3,79 14,40±2,79 6,81±1,03

Table 2. Average Motility of Individual Bali Bull in Various Diluent Combinations during Cooling

Treatment	Motility (%)								
	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9
P0	72,42 ± 1.24	65,55± 1.24	62,70 ± 0.92	59,51 ± 0.72	55,64 ± 0.82[b]	50,03 ± 0.51 [b]	46,98 ± 1.10 [b]	42,41 ± 1.72 [b]	32,30 ± 0.21 [b]
P1	70,64 ± 1.81	65,54 ± 0.76	63,32 ± 0.78	51,14 ± 0.43	42,26 ± 1.40[a]	23,06 ± 0.42 [a]	14,78 ± 0.21 [a]	10,28 ± 0.20[a]	9,32 ± 0.00[a]
P2	70,43 ± 1.21	64,5 ± 0.42	62,17 ± 0.54	53,57 ± 0.08	43,19 ± 1.34[a]	27,21 ± 0.14 [a]	16,29 ± 0.11 [a]	13,41 ± 0.26[a]	11,21± 0.00 [b]

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P3	69,41 ± 1.21	67,4 ± 0.43	63,2 ± 0.54	45,61 ± 1.06	42,16 ± 1.37 [a]	20,28 ± 0.43 [a]	12,9 ± 0.21 [a]	10,51 ± 0.26 [a]	9,10± 0.00 [a]

Note: Superscripts on different letters indicate very significant differences (P < 0.01)

Table 3. Average Membrane Integrity after Preservation at 5° C

Treatment	Integrity Of Sperm Cell (%)		
	Fresh	H-1	H-5
P0 (CEP-2 + KT 10%)	87,31 ± 2,31	85,10 ± 0,07	69,14± 0,05[b]
P1 (EBM+AK+KT 15%)	87,31 ± 2,31	85,06 ± 1,03	60,12 ± 0,03[a]
P2 (EBM+AK+KT 15%+ Fruk 1 mg/ml)	87,31 ± 2,31	85,04 ± 1,08	64,04 ± 0,06[a]
P3 (EBM+AK+KT 15%+ Fruk 2 mg/ml)	87,31 ± 2,31	85,01 ± 1,11	60,03 ± 0,04[a]

Note: Superscripts on different letters indicate very significant differences (P < 0.01)

Table 4. Average Not yet Capacitated After Preservation at 5° C

Treatment	Not yet Capacitated (%)		
	Fresh	H-1	H-5
P0 (CEP-2 + KT 10%)	85,22 ± 2,14	83,51 ± 1,11	66,24± 0,10[a]
P1 (EBM+AK+KT 15%)	85,22 ± 2,14	83,43 ± 1,06	62,43 ± 0,08[b]
P2 (EBM+AK+KT 15%+ Fruk 1 mg/ml)	85,22 ± 2,14	83,26 ± 1,06	64,16 ± 0,06[b]
P3 (EBM+AK+KT 15%+ Fruk 2 mg/ml)	85,22 ± 2,14	83,08 ± 1,14	63,13 ± 0,02[b]

Note: Superscripts on different letters indicate very significant differences (P < 0.01)

Table 5. Average Capacitation After Preservation at 5° C

Treatment	Capacitation of Sperm (%)		
	Fresh	H-1	H-5
P0 (CEP-2 + KT 10%)	13,51±2,60	11,34±1,19	12,66±0,13 [b]
P1 (EBM+AK+KT 15%)	13,51±2,60	14,20±1,20	15,18±0,10[a]
P2 (EBM+AK+KT 15%+ Fruk 1 mg/ml)	13,51±2,60	13,88±1,21	14,70±0,04[a]
P3 (EBM+AK+KT 15%+ Fruk 2 mg/ml)	13,51±2,60	14,34±1,22	15,11±0,07[a]

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Table 6. Average Acrosome Reaction After Preservation at 5° C

Treatment	Acrosome Reaction (%) ± SD		
	Fresh	H-1	H-5
P0 (CEP-2 + KT 10%)	5,70±2,18	5,50±1,12	8,12±0,09[a]
P1 (EBM+AK+KT 15%)	5,70±2,18	6,30±1,10	13,10±0,13[b]
P2 (EBM+AK+KT 15%+ Fruk 1 mg/ml)	5,70±2,18	5,22±1,29	10,44±0,14[b]
P3 (EBM+AK+KT 15%+ Fruk 2 mg/ml)	5,70±2,18	6,13±1,15	10,11±0,18[b]

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ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

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ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

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Conclusion

The diluent formulation containing 10% CEP-2 and egg yolk proved superior to that consisting of red fruit extract and coconut water. This conclusion is based on the observation that the former resulted in a higher proportion of spermatozoa with intact membranes and no capacitation, whereas capacitation and acrosome reaction remained low. Furthermore, the diluent formulation containing red fruit extract and coconut water was found to maintain the acrosome status above 50% after five days of shelf life.

Acknowledgment

The Authors gratefully acknowledge the support of the LP2M University of Jember as the research funding provider.

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