Original article

Coconut water combined with purebred chicken egg yolk as an alternative extender for Pote buck semen

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ABSTRACT

Artificial insemination (AI) is a key strategy to improve livestock genetics and ensure the quality of preserved semen, which is crucial to the success breeding programs. Extending semen storage time while maintaining its quality is a significant challenge. This research investigated the use of coconut water and egg yolk citrate extender to preserve Pote buck spermatozoa at 5°C. Coconut water, with its rich content of carbohydrates, proteins, vitamins, and antioxidants, and egg yolk, which contained lecithin, were selected for their potential to maintain spermatozoa quality during storage. The experiment involved three treatment groups, T0, T1, and T2, where semen were diluted 10 times each in egg yolk citrate, coconut water, and a combination of egg yolk citrate (20%) and coconut water. Spermatozoa motility, viability, intact plasma membrane, abnormality, and malondialdehyde (MDA) levels were assessed daily for five days. The results showed no significant difference between T0 and T1 ($p > 0.05$). However, T2 showed the highest progressive motility, viability, and intact plasma membrane percentages, in addition to the lowest spermatozoa abnormality and MDA levels. These findings indicate that the combination of coconut water and egg yolk citrate was the most effective extender for preserving Pote buck semen at 5°C, which contributed to the broader goals of improving sustainable livestock production and supporting food security by improving genetic diversity.

Keywords: abnormality, chilled storage, food security, livestock production, MDA, spermatozoa quality

INTRODUCTION

Pote goats, which are native to the Bangkalan district, are characterized by their thick white fur, small body size similar to Ettawa goats, drooping but not overly long earlobes, pink lips, and a curled tail. Both male and female Pote goats have beards, and both are valuable for meat and milk production (Rohman *et al*., 2023). Despite their potential, the Pote goat mating system in Bangkalan is still largely natural. However, to increase productivity and population, artificial insemination (AI) technique can be employed. AI could utilize fresh, diluted, or frozen semen (Susilowati *et al*., 2021). An important factor influencing the success of AI was semen quality, which was determined by the preservation technique, extender composition, and type and concentration of cryoprotectant used. Proper

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semen preservation was essential to optimize the use and distribution of superior male genetics throughout the breeding programs, particularly through cold storage (Stuart *et al*, 2019).

Cold storage of semen was intended to suppress spermatozoa metabolism, allowing the semen to remain viable for AI programs for up to five days (Allai *et al*., 2018). However, during the preservation process, spermatozoa were exposed to external air containing oxygen, resulting in the creation of free radicals, commonly referred to as reactive oxygen species (ROS) (Khan *et al*., 2017). Free radicals, which were also known as ROS, are very dangerous for spermatozoa because they could cause damage to the plasma membrane (Sabeti *et al*., 2016; Mustofa *et al*., 2021). Semen needed to be supported with an extender that fulfil the requirements to meet the physical and chemical needs of spermatozoa during the preservation process so that it could maintain its viability (Susilowati *et al*, 2021b). In addition to increasing volume, the function of extenders was to reduce the toxic potential of seminal plasma. The extender used must be able to maintain the ionic stability of the plasma membrane so that it remained normal (Susilowati *et al*., 2019).

The composition of the extender greatly determined the quality of spermatozoa in chilled stored semen for AI. Young coconut water could be used as an extender for livestock semen because it contained carbohydrates, vitamins, minerals, protein, amino acids, and fatty acids and also contains vitamin C. Apart from that, coconut water could be an alternative extender that was easy to obtain and easy to adapt to the environment. The carbohydrate content was 3.8 grams, protein 0.2 grams, and fat 0.1 grams. The function of egg yolk was to maintain the integrity of the spermatozoa envelope and prevent cold shock because it contained lecithin. Apart from that, egg yolk also contained glucose as an energy source, several protein substances and vitamins as well as egg yolk fat which could limit the movement of spermatozoa and suppress the energy breakdown process (Susilowati *et al*., 2019). Currently, frozen semen from Pote goat studs was unavailable, indicating a significant gap in the application of AI in this breed. Therefore, this study was designed to evaluate the effectiveness of using a combination of young coconut water and purebred chicken egg yolk as an extender for preserving Pote buck semen. The objective of this study was to contribute to the preservation of the breed's genetic resources, address gaps in semen preservation methods and provide sustainable solutions to increase livestock production.

MATERIALS AND METHODS

This research was carried out in Patenteng village, Modung district, Bangkalan city. This study used two superior male Pote goats aged approximately 3-4 years with a body weight of 26-27 kg. Semen collection was carried out twice a week. The extender used were young green coconut water, egg yolk from purebred chickens.

Pote bucks were adapted for one week and given forage and concentrate. Before use, bucks were also trained to collecting their semen using an artificial vagina. The collected semen was then evaluated both macroscopically and microscopically. Macroscopic evaluation involved checking the volume, pH, consistency, color, and odor, while microscopic examination included mass movements, individual movements, viability, and abnormalities. Samples were processed for preservation if the percentage of individual motility and viability were more than 70% and the mass movement was at least +2.

Extender preparation

The egg shell was cleaned with 70% alcohol cotton, the egg shell was broken in the air cavity using tweezers, and the egg white liquid was discarded. The intact egg yolk was transferred onto filter paper and rolled to eliminate any residual egg white. The vitelline membrane was ripped, allowing the egg yolk to be transferred into a measuring cylinder. A citrate solution was then introduced (2.9 grams of sodium citrate mixed with 100 mL of distilled water, heated to 92-95°C, and subsequently cooled to room temperature) in a ratio of 1:1.

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Young coconut water was obtained from young green coconut. The pH of young coconut water was checked and 20% egg yolk citrate was added, and antibiotics were also added (1000 IU/mL Penicillin and 1 mg/mL Streptomycin). Semen were diluted 10 times in egg yolk citrate extender (T0), young coconut water extender (T1), and a combination of egg yolk citrate and young coconut water extender. The pH of each diluted semen were measured then the tubes were closed and placed in a beaker containing clean water, and stored in a refrigerator (5°C). Spermatozoa from the three treatment groups was checked every day for the quality. Regular checks were carried out every day to determine the quality of semen that could still be used for AI. Observations of spermatozoa included progressive individual movement, viability, abnormalities, intact plasma membrane, and Malondialdehyde (MDA) levels.

Examination of spermatozoa progressive individual movements

Progressive movement of individual spermatozoa was examined by mixing 10 µL of semen with 10 µL of homogenized physiological NaCl on an object glass. The mixture was then cover slipped and examined under a phase contrast microscope with a magnification of 400x. Spermatozoa that moved progressively or moved forward were recorded (Susilowati *et al*., 2019).

Examination of spermatozoa viability

Examination of the viability or life and death of spermatozoa was carried out by dripping a drop of semen onto an object glass and adding one drop of eosin nigrosine dye, homogenizing it, and making a smear. Next, the smear was fixed over a flame and then observed using a phase contrast microscope with a 400x magnification. Live spermatozoa appeared transparent or clear in the head area, while dead spermatozoa showed a reddish or purplish color in the head area (Pahlevy *et al*, 2022).

Examination of spermatozoa abnormalities

Examination of spermatozoa abnormalities was carried out by dripping one drop of semen onto an object glass and adding one drop of eosin nigrosine dye, homogenizing it, and making a topical preparation. Next, it was fixed over a flame and then examined under a phase contrast microscope at 400x magnification. Spermatozoa were considered abnormal if the spermatozoa heads were double, small, or large and the spermatozoa tails were double, or circular (Pahlevy *et al*., 2022).

Examination of spermatozoa intact plasma membrane

Intact plasma membranes were examined using a hypoosmotic swelling (HOS) solution. One hundred µL of semen was mixed with 1 mL of HOS solution (consisting of 13.51 grams of fructose, and 7.35 g of sodium citrate in 1000 mL of distilled water), incubated at 25°C for an hour, then 15 µL of the sample was applied to a glass slide, covered with a cover slip, and then observed under a phase contrast microscope at a 400x magnification. Spermatozoa with a bulging neck, or a coiled tail meant that the spermatozoa had intact membranes, while spermatozoa whose neck-to-tail were not bulging meant that the membranes were damaged (Susilowati *et al*., 2024).

Malondialdehyde (MDA) level examination

MDA levels were assessed using a modification of the Thiobarbituric Acid (TBA) test method. One hundred µL of MDA standards each with concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8 µg/mL was added with 550 µL of distilled water, 100 µL of 20% trichloroacetic acid, and homogenized for 30 seconds. Then 250 µL of 1 N HCl, and 100 µL of 1% sodium thiobarbiturate were added, and homogenized. Then the mixture was centrifuged at 500 rpm for 10 minutes. The supernatant was heated in a water bath at 100°C for 30 minutes and then allowed to cool to room temperature. The color intensity was measured with a spectrophotometer at a wavelength of 533 nm. Standard absorbance values and MDA standard curves were obtained. MDA levels of

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samples were obtained by converting the absorbance measurement results with the standard curve values of pure MDA standards in various concentrations. Next, the resulting value of the standard curve is multiplied again by the dilution factor used. MDA concentration was measured based on the number of µg or nmol or ppm MDA in each mL of spermatozoa suspension (Susilowati *et al*., 2024).

Data analysis

The data were examined using one-way analysis of variance, followed by Duncan's multiple range test. The statistical evaluation was performed at a 95% significance level with the aid of Statistical Product and Service Solutions (SPSS, version 23; IBM Corp., USA).

RESULTS

The quality of fresh Pote buck semen that would be used can be seen in Table 1.

Table 1 Macroscopic and microscopic examination of fresh Pote buck semen

volume (mL)	1.37 ± 0.06
color	creamy (normal)
pH	6 – 7
consistency	thick (normal)
concentration (million/mL)	1933.67 ± 51.03
mass movement	+++ (normal)
progressive motility (%)	83.33 ± 2.89
viability (%)	88.33 ± 1.52
abnormality (%)	2.12 ± 0.03
intact plasma membrane	72.12 ± 0.05

Fresh Pote buck semen that would be processed or stored must have a percentage of progressive motility and viability of more than 70% and abnormalities of less than 15% (Pasha *et al*., 2022). The data on the quality of fresh semen from Pote bucks can be seen in Table 1. The semen collected from 2 Pote bucks was creamy white in color, had a thick consistency, and a pH range of 6-7. The volume averaged 1.37 ± 0.06 mL, with a concentration of 1933.67 ± 51.03 million/mL, and mass motility at +++ (indicative of robust spermatozoa movement). Additionally, the progressive motility averaged 83.33 ± 2.89%, viability at 88.33 ± 1.52%, intact plasma membrane at 72.12 ± 0.05%, and abnormalities at 2.12 ± 0.03%. These results confirm that, the quality of Pote buck semen met the requirements to be used in this study.

Table 2 Progressive spermatozoa motility (%) stored at 5°C in different extenders

	day-1	day-2	day-3	day-4	day-5
T0	80.00 ± 0.00 ^{b}	72.22 ± 3.28 ^{b}	63.89 ± 4.04 ^{b}	53.89 ± 4.30 ^{b}	43.33 ± 2.98 ^{b}
T1	80.83 ± 1.39 ^{b}	73.8 ± 2.74 ^{b}	63.33 ± 3.34 ^{b}	52.06 ± 3.86 ^{b}	44.17 ± 2.30 ^{b}
T2	83.33 ± 1.49 ^{a}	78.89 ± 2.51 ^{a}	72.78 ± 4.17 ^{a}	63.89 ± 3.10 ^{a}	54.46 ± 1.72 ^{a}

different superscripts in the same column indicate significant differences ($p < 0.05$); T0: semen was diluted in egg yolk citrate extender; T1: semen was diluted in coconut water extender; T2: semen was diluted in a combination of egg yolk citrate (20%) and coconut water extender; all dilutions: 10x.

Table 3 Viability of spermatozoa stored at 5°C in different extenders

	day-1	day-2	day-3	day-4	day-5
T0	82.00 ± 0.20 ^{b}	76.32 ± 3.27 ^{b}	68.87 ± 3.04 ^{b}	59.85 ± 3.30 ^{b}	50.33 ± 2.88 ^{b}
T1	82.82 ± 1.29 ^{b}	77.13 ± 2.64 ^{b}	68.13 ± 3.34 ^{b}	59.26 ± 3.46 ^{b}	51.07 ± 2.20 ^{b}
T2	86.33 ± 1.49 ^{a}	82.89 ± 2.31 ^{a}	77.78 ± 4.20 ^{a}	69.89 ± 3.10 ^{a}	59.43 ± 1.62 ^{a}

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different superscripts in the same column indicate significant differences (p <0.05); T0: semen was diluted in egg yolk citrate extender; T1: semen was diluted in coconut water extender; T2: semen was diluted in a combination of egg yolk citrate (20%) and coconut water extender; all dilutions: 10x.

Table 4 Intact plasma membrane of spermatozoa (%) stored at 5°C in different extenders

	day-1	day-2	day-3	day-4	day-5
T0	66.01 ± 0.20 [b]	64.32 ± 3.27 [b]	52.87 ± 3.04 [b]	46.85±3.30 [b]	39.33 ± 2.58 [b]
T1	65.62 ± 1.29 [b]	63.33 ± 2.64 [b]	53.23 ± 3.34 [b]	47.26±3.36 [b]	40.17 ± 2.36 [b]
T2	75.33 ± 1.45 [a]	72.89 ± 2.31 [a]	67.78 ± 4.25 [a]	58.69±3.10 [a]	49.43 ± 1.42 [a]

different superscripts in the same column indicate significant differences (p <0.05); T0: semen was diluted in egg yolk citrate extender; T1: semen was diluted in coconut water extender; T2: semen was diluted in a combination of egg yolk citrate (20%) and coconut water extender; all dilutions: 10x.

Table 5 Abnormal spermatozoa (%) stored at 5°C in different extenders

	day-1	day-2	day-3	day-4	day-5
T0	5.05 ± 0.05 [b]	6.32 ± 3.27 [b]	6.37 ± 1.04 [b]	6.75 ± 0.20 [b]	7.33 ± 2.18 [b]
T1	4.72 ± 1.27 [b]	6.33 ± 1.64 [b]	5.23 ± 2.34 [b]	6.26 ± 2.36 [b]	7.10 ± 1.05 [b]
T2	2.23 ± 1.35 [a]	3.80 ± 2.01 [a]	3.75 ± 1.25 [a]	4.39 ± 3.10 [a]	4.43 ± 1.42 [a]

different superscripts in the same column indicate significant differences (p <0.05); T0: semen was diluted in egg yolk citrate extender; T1: semen was diluted in coconut water extender; T2: semen was diluted in a combination of egg yolk citrate (20%) and coconut water extender; all dilutions: 10x.

Table 6 MDA levels (nmol/mL) of spermatozoa stored at 5°C in different extenders

	day-1	day-2	day-3	day-4	day-5
T0	1779.43 ± 45.16 [b]	1887.40 ± 35.50 [b]	1890.34 ± 40.35 [b]	1905.45 ± 30.03 [b]	1935.25 ± 20.04 [b]
T1	1705.18 ± 20.25 [b]	1760.28 ± 25.15 [b]	1898.56 ± 25.20 [b]	1953.36 ± 20.18 [b]	1980.25 ± 15.38 [b]
T2	1235.20 ± 10.05 [a]	1255.10 ± 05.10 [a]	1289.34 ± 17.05 [a]	1302.07 ± 15.00 [a]	1326.15 ±2 0.07 [a]

different superscripts in the same column indicate significant differences (p <0.05); T0: semen was diluted in egg yolk citrate extender; T1: semen was diluted in coconut water extender; T2: semen was diluted in a combination of egg yolk citrate (20%) and coconut water extender; all dilutions: 10x.

The study's findings indicated that progressive motility, life and death, abnormalities, intact plasma membranes, and MDA levels of spermatozoa from the 3 groups showed that the best was T2 (coconut water + egg yolk). In contrast, no significant differences were observed between groups T0 and T1 (p >0.05). As storage time increased, there was a decline in progressive motility, viability, and intact plasma membrane percentages, while the percentages of abnormalities and MDA levels increased.

DISCUSSION

The evaluation of buck semen quality was essential to determine its suitability for cold storage. The initial assessment included both macroscopic and microscopic parameters. Macroscopically, semen characteristics such as color, odor, pH, volume, and consistency were evaluated, while microscopically, parameters such as motility, viability, membrane integrity, and spermatozoa abnormalities were examined (Junaedi *et al*., 2024).

Semen color is a reflection of semen consistency. Semen that has a thick consistency

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has a darker color (Athalla *et al*., 2023). The average volume of buck semen collected in this research was in the normal range. Buck semen collected using an artificial vagina had a volume ranging from 0.5-2 mL (Susilowati *et al*, 2023). The volume of buck semen ejaculated could be influenced by factors such as breed, body size, age, and diet (Syarifuddin *et al*., 2022). The results of the acidity degree (pH) examination showed an average of 6.8, aligned with the fertile range for buck semen, which was between 5.9 and 7.0 (Wurlina *et al*., 2020). The microscopic examination results showed an average mass movement of +++, with a progressive spermatozoa motility of 83.33 ± 2.89%, viability of 88.33 ± 1.52%, and intact plasma membrane percentage of 72.12 ± 0.05%. The abnormalities percentage was low, at 2.12 ± 0.03%. These values were consistent with fertile buck semen characteristics (Junaedi *et al*., 2024). According to Pasha *et al*. (2022), ejaculates suitable for further processing should exhibit +++ mass movement and have motility and viability percentages exceeding 70%. The intact plasma membrane percentage obtained in this study also met the necessary criteria, as semen with less than 60% intact plasma membrane was categorized as infertile (Siregar *et al*., 2023). The spermatozoa concentration was 1933.67 ± 51.03 million/mL, which was slightly below the ideal concentration for AI, which should exceed 2,000 million/mL (Susilowati *et al*., 2021). Based on these macroscopic and microscopic examination results, the fresh Pote buck semen used in the study met the criteria for further processing and potential use in AI.

Motility was one of the parameters to determine the quality of spermatozoa. Motility value was an important thing that can influence the fertility level of spermatozoa in fertilizing the ovum (Dcunha *et al*., 2022, Rosyada *et al*., 2023). Viability was also a parameter to determine the grade of spermatozoa. It is important to see the viability or survival value of spermatozoa to determine how long spermatozoa remain alive in the female reproductive tract (Eckel *et al*., 2017).

The reliability of the spermatozoa plasma membrane was necessary to ensure their survival and success in fertilizing the egg (Sitepu and Marisa, 2019). Apart from functioning to protect cell organelles from mechanical damage, the plasma membrane also played an important role as a good filter for the exchange of intra- and extracellular substances that were maintained in metabolic processes (Susilowati *et al*., 2021). If damage occured to the membrane, it would modulate the formation of ROS by spermatozoa. ROS could damage cells or molecules around them by oxidizing or reducing electrons from other molecules around them (Mauchart *et al*., 2023) and it had been proven that ROS could cause cell dysfunction (Agarwal *et al*., 2003) through changes in the function of structural proteins, chains DNA and cell membranes so that cell integrity was disrupted (Juan *et al*., 2021). If one form of ROS, namely hydroxyl radicals, reacted with docosahexaenoic acid (DHA), a chain reaction called lipid peroxidation would occur. The final result of this chain reaction was the breaking of the fatty acid chain into an aldehyde compound, namely malondialdehyde (MDA). If higher levels of lipid peroxidation occured, MDA levels would increase (Yui *et al*., 2023).

The findings indicated that the quality of spermatozoa at 5°C storage from the first to the fifth day was the best in the T2 group. This was because the egg yolk used as an extender contained lecithin or lipoprotein which could preserve the integrity of the spermatozoa envelope from cold temperatures and also contained glucose as an energy source (Gitayana *et al*, 2023). Young coconut water contained carbohydrates, protein, vitamin C, and antioxidants. Coconut water also had antibacterial property (Vicente *et al*., 2018). The results of the study from the first to the fifth day of storage showed a decrease in the quality of spermatozoa in all extender groups, this was due to continuous metabolism and the formation of ROS. ROS could harm the plasma membrane. The spermatozoa plasma membrane was susceptible to lipid peroxidation where unsaturated fatty acids were broken down as the largest component of the plasma membrane that encased cells and organelles (Sabeti *et al*., 2016).

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This lipid peroxidation chain reaction continued continuously until it finally caused changes in membrane function which resulted in a decrease in spermatozoa motility (Qamar *et al*, 2023). During semen storage, there would be damage to protein decomposition in the spermatozoa plasma membrane resulting in protein denaturation in the lipoprotein layer caused by peroxidation of the spermatozoa membrane (Silvestre *et al*., 2021). Plasma membrane damage could be minimized by the presence of antioxidants and vitamin C contained in coconut water (Triandari *et al*., 2024).

Overall, the use of coconut water combined with egg yolk citrate in the extender showed a significant improvement of Pote buck semen quality during storage at 5°C. The findings suggest that this combination might be an effective alternative for maintaining semen quality during cold storage, offering potential benefits for AI programs in goats. Further research could explore the specific mechanisms by which coconut water contributed to improve spermatozoa preservation, as well as its application in different breeds and species.

CONCLUSION

This study concluded that the use of young coconut water and purebred chicken egg yolk as an extender was effective in maintaining the quality of Pote buck semen at chilled temperature for up to five days. This combination maintained key parameters such as progressive motility, viability, and intact plasma membrane integrity, while minimizing abnormalities and MDA levels. These results indicate that this extender is a feasible solution for enhancing the preservation of Pote buck semen and addressing current limitations in semen storage methods. This approach promises to improve artificial insemination programs and advance the management of Pote goat genetic resources. Further research is recommended to explore the specific mechanisms of the extender efficacy and its applicability across different breeds and species.

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AUTHOR'S CONTRIBUTIONS

Tatik Hernawati (TH), Suherni Susilowati (SS), Widya Paramita Lokapirnasari (WPL), Tri Wahyu Suprayogi (TWS), Zulfi Nur Amrina Rosyada (ZNAR).

TH: Formulated the idea and created the framework for this manuscript. TH, TWS: acquisition data. SS, WPL: interpretation and analysis data. TH, SS: manuscript drafting. TH, TWS, ZNAR: Carefully examined and revised the manuscript for its intellectual content. All authors reviewed and endorsed the final version.

CONFLICTS OF INTEREST

The authors states that they have no conflict of interests.

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