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RESEARCH ARTICLE

Extenders in Sperm Sexing: Boosting Motility and Viability in Barbados Blackbelly Rams with Coconut Water and Egg Yolk

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Abstract The application of natural extenders like egg yolk and coconut water for sperm sexing in livestock remains underexplored, particularly for species like the Barbados Blackbelly ram. This study evaluates the effectiveness of various extenders in the egg white sedimentation (EWS) approach and their impact on the motility, viability, and morphology of Barbados Blackbelly ram's sperm. Semen samples were collected from Barbados Blackbelly rams (n=5), and assessed through macroscopic (color, viscosity, volume) and microscopic (motility, progressive motility, viability and morphology) evaluations before and after sexing. The collected semen was then subjected to sexing albumin gradient (two-layer albumin: 10% and 30%) prepared with different extenders: Control (C: tris-based solution), Treatment 1 (T1: boiled mature coconut water), Treatment 2 (T2: fresh mature coconut water), Treatment 3 (T3: boiled young coconut water), Treatment 4 (T4: fresh young coconut water), Treatment 5 (T5: chicken egg yolk), and Treatment 6 (T6: duck egg yolk). Separated X (top of the gradient) and Y sperm (bottom of the gradient) were extracted after 20-minute incubation, then the samples were centrifuged at 1500 rpm for five minutes before they were analyzed. The study demonstrates that natural based extenders, including coconut water and egg yolk, significantly enhance sexed sperm motility, viability, and morphology in Barbados Blackbelly rams across various treatment groups. Treatment groups T3 (young fresh coconut water) and T4 (boiled coconut water) improve sperm motility and velocity due to their rich content of antioxidants, sugars, and electrolytes, while T6 (duck egg yolk) helps stabilize sperm membranes with phospholipids like lecithin and cholesterol. Of the extenders tested, T4 (boiled coconut water) showed the most successful extender tested, produced the most increases in sperm quality, motility, and viability. These extenders offer small-scale farmers an affordable way to increase access to sperm sexing and support sustainable livestock reproduction.

Keywords: Sperm quality, egg white sedimentation, natural extenders, coconut water, Barbados blackbelly rams.

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Introduction

The ability to control offspring sex in farm animals is generating significant interest in agricultural society with its potential for transforming cattle and small ruminant production. Optimum management of cattle

and small ruminant sex ratio, for example, brings a range of dividends, such as welfare gain, rapid genetic selection, and maximizing efficiency in utilizing resources [1] [6]. By regulating male and female proportion, producers can manage to position herds in relation to demand in the marketplace, save maintenance costs, and, in the long term, maximize productivity.

Several methods have been developed for sperm sexing, but most, such as flow cytometry, have high cost, complex processes, and technical expertise requirements, and, for that reason, cannot become accessible for small farms. On the other hand, egg white sedimentation (EWS) is a cost-effective but efficient alternative. EWS takes advantage of an albumin gradient to naturally sort X- and Y-bearing sperm, and a less complex and less expensive sperm technology with potential for future use in the agricultural sector [2] [16].

Combining sexed semen with artificial insemination (AI) generates added value, with farmers having the ability to predetermine offspring gender according to financial aims. For instance, female calves can be desired in dairies, and male calves in the beef industry [3] [12]. By regularly isolating X and Y sperm, producers can make use of progeny testing in a more efficient manner, reduce pregnancies for breeding stock appraisal, and overall, enhance herd genetics [4]. In addition, using AI with sexed semen can reduce disease transmission danger, but contribute even further to biosecurity and herd welfare [10].

Recently, there have been heightened concerns regarding utilizing extenders of a natural origin in AI programs, in that such extenders could potentially enhance sperm viability and conservation. There are high concentrations of bioactive compounds and antioxidants in such extenders, such as egg yolk and coconut water, and can, therefore, improve sperm quality during sexing [18][30]. Minimal study has been conducted on the application of these extenders in EWS for sperm sexing, particularly in ovine species such as the Barbados Blackbelly ram. This study aims to assess how different types of natural extenders of coconut water (young vs. mature; fresh vs. boiled) and egg yolk sources (chicken vs. duck) used in extenders influence the motility, viability, morphology, and overall performance of sperm sorted by egg white sedimentation (EWS) in Barbados Blackbelly rams.

This study addresses such a loophole with an examination of egg yolk and coconut water extenders' effectiveness in terms of viability, motility, and shape of sexed sperm in Barbados Blackbelly rams. By testing such naturally derived extenders in the EWS model, such a study will make significant contribution towards cost-effective and environmentally friendly options for enhancing breeding in animals.

Materials and Methods

Experimental Animals

Five Barbados Blackbelly rams (age: 2 – 3 years, and body score condition (BCS): 3.0 – 3.5) were selected for use in this study based on their fertility profiles. Rams were kept under proper care and nutrition in agreement with farms' routine nutrition regimes. Sperm collection using an artificial vagina technique and processing with a two-layer albumin gradient with variable extenders were performed.

Selection of Coconut

The Malayan tall coconuts (*Cocos nucifera*) were chosen based on characteristics listed in Table 1 according to [5][35].

Table 1. Characteristics of young and mature coconut [5][35]

Characteristic	Young Coconut	Mature Coconut
Colour	Green (light to dark)	Brown (light to dark)
Surface	Smooth, slightly glossy	Rough, with visible husk fibres
Husks	Thick, tightly adhering to the nut	Thinner, may detach easily
Eyes (germination points)	Soft, yielding to pressure	Hard, resistant to pressure
Sound	Hollow, swishing sound when shaken	Dull thud when shaken
Weight	Lighter due to higher water content	Heavier due to thicker flesh and less water

Preparation of Treatment

Coconut Water

Both mature and young coconut water were used, with treatments involving boiling and non-boiling [6] [33]. To control pH, 0.6 g of NaHCO3 was added after boiling to achieve a pH of 7.0.

Egg yolk

Egg yolk was separated from albumin, filtered, and mixed with a Tris-based solution to achieve a consistent concentration. Both chicken and duck eggs were cleaned, and their yolks separated from the whites. To prepare 1000 ml of semen extender, 20 ml of egg yolk was required. The Tris-based solution was formulated by dissolving 3.8 g of Tris powder, 2.1 g of citric acid, 3.0 g of penicillin, and 5.0 g of streptomycin in 100 ml of distilled water, adjusting the pH to a range of 6.5–6.7. The egg yolks were then mixed with the Tris extender in a ratio of one part egg yolk to four parts Tris solution (4:1 Tris to egg yolk) [4].

Gradient Solution

Egg whites from laying hen were filtered to separate the albumen from the yolk which is essential for the gradient solution [16] [25]. According to the treatments listed in Table 2, egg albumin was added to the extender. Seven treatments, which are Control (C), Treatment 1 (T1), Treatment 2 (T2), Treatment 3 (T3), Treatment 4 (T4), Treatment 5 (T5), and Treatment 6 (T6) were prepared for this study.

Table 2. Different treatment extenders (media) for sperm sexing using egg white sedimentation (EWS) method

Treatment	Type of extender
Control	Tris-based solution
1	Fresh mature coconut water-based extender
2	Boiled mature coconut water-based extender
3	Fresh young coconut water-based extender
4	Boiled young coconut water-based extender
5	Chicken egg yolk extender
6	Duck egg yolk extender

Semen Collection

The selected rams were initially undergoing sperm-collecting training using artificial vagina (AV) method [23][25]. The collection of semen took place in the morning, the AV was filled with warm water that is 37°C and air. KY jelly was applied as a lubricant at the vaginal entrance to facilitate the process of semen collection process, [17]. Once was collected, the semen was examined under a microscope for mass motility, total motility, progressive motility, viability, abnormalities, and concentration, as well as colour, volume, and pH [3].

Sperm Evaluation and Analysis

Sperm analysis is crucial for determining male fertility. fresh semen from Barbados Blackbelly rams must have a minimum concentration of 1500 million spermatozoa per milliliter, 70% motility, 80% viability, and mass motility ++ before being sexed [3].

Semen Colour

The colour of the semen was evaluated and categorized as 1 = clear/watery, 2 = cloudy/bloody, 3 = milky, and 4 = creamy [14].

Semen pH

The pH of the semen was ascertained using the pH meter. Before being placed into a tube featuring semen, the electro rod was cleaned and rinsed with sterile water [14].

Sperm Motility and Concentration

The sample is loaded into the IVOS instrument (IVOS® II Sperm Analyzer. (n.d.). Hamilton Thorne, USA) with using a pipette, loading a small volume (typically 5-10 µL) of the sample into the analysis chamber or slide. It must avoid introducing air bubbles. The software uses the microscope to capture digital images or videos of the sperm. The system then identifies and counts individual sperm cells based on their size, shape, and movement patterns. Following this analysis, the software generates a report detailing various sperm parameters, including concentration (number of sperm per unit volume), motility (percentage of motile sperm), and velocity (speed of sperm movement) [4].

Sperm Viability

Smear preparation with Hancock solution was used to calculate the proportion of live and dead sperm [14]. The calculation is as follows:

$$viability = \frac{Total\ live\ spermatozoa}{\text{Total spermatozoa observed}} \times 100$$

Sperm Abnormality

The sperm abnormalities were examined under a 1000x compound microscope using the same slides as in the study viability. Sperm abnormalities are classified into two categories: primary abnormalities, which include sperm without a head, double tail, damaged acrosome, and spiral tail, and secondary abnormalities, which include sperm without a tail, damaged tail, matured tail, or no tail at all [14].

Sperm Sexing Using Egg White Albumin

Based on [25], this method involved mixing with the extender in a centrifuge tube. The sexing medium was prepared by separating egg white from egg yolk creating two layers of albumin: a 10% and a 30% albumin gradient. These gradients were mixed in another tube to form the gradient solution (Table 3). Both gradient solutions were mixed in another centrifuge tube, forming gradient solution consisting of 10% of the upper fraction and 30% of lower fraction of the gradient. The sperm and gradient mixture were incubated in a water bath at 34°C for 20 minutes. After incubation, sperm were extracted and centrifuged at 1500 rpm for five minutes. In this experiment, seven types of extenders were used: Control (C: tris-based solution), Treatment 1 (T1: boiled mature coconut water), Treatment 2 (T2: fresh mature coconut water), Treatment 3 (T3: boiled young coconut water), Treatment 4 (T4: fresh young coconut water), Treatment 5 (T5: chicken egg yolk), and Treatment 6 (T6: duck egg yolk). During the post-incubation period, the sexed sperm were centrifuged for five minutes and analysed for their quality in terms of motility, viability, and concentration after the samples [31]. The microscopic evaluation (motility, viability, and morphology) of X and Y sperm was then carried out using the Hancock stain [17].

Table 3. The percentage of albumin and extender in 10 ml of media solution

Tube	Percentage of solution	Prepare of 10 ml of solution
1	10% albumin and 90% extender	1 ml albumin and 9 ml extender
2	30% albumin and 70% extender	3 ml albumin and 7 ml extender

Statistical Analysis

Motility, viability, and morphology of X- and Y-bearing sperm were analyzed using microscopy and IVOS® II Sperm Analyzer. Results were statistically evaluated using one-way ANOVA with Tukey's test ($P<0.05$) to determine significant differences. All data analyses were conducted using Minitab 21, ensuring consistency in statistical evaluation.

Results and Discussion

The results demonstrate a significant improvement in sperm quality metrics such as motility, viability, morphology, and purity when natural extenders like coconut water and egg yolk are used in sperm sexing for Barbados Blackbelly rams.

Motility and Velocity

Table 4. Total motility, progressive motility and velocity distribution (mean±SEM) of X and Y sperm after EWS sperm sexing using different treatments: Control (C: tris based solution), Treatment 1 (T1: fresh mature coconut water), Treatment 2 (T2: boiled mature coconut water), Treatment 3 (T3: fresh young coconut water), Treatment 4 (T4: boiled young coconut water), Treatment 5 (T5:chicken egg yolk) and Treatment 6 (T6: duck egg yolk)

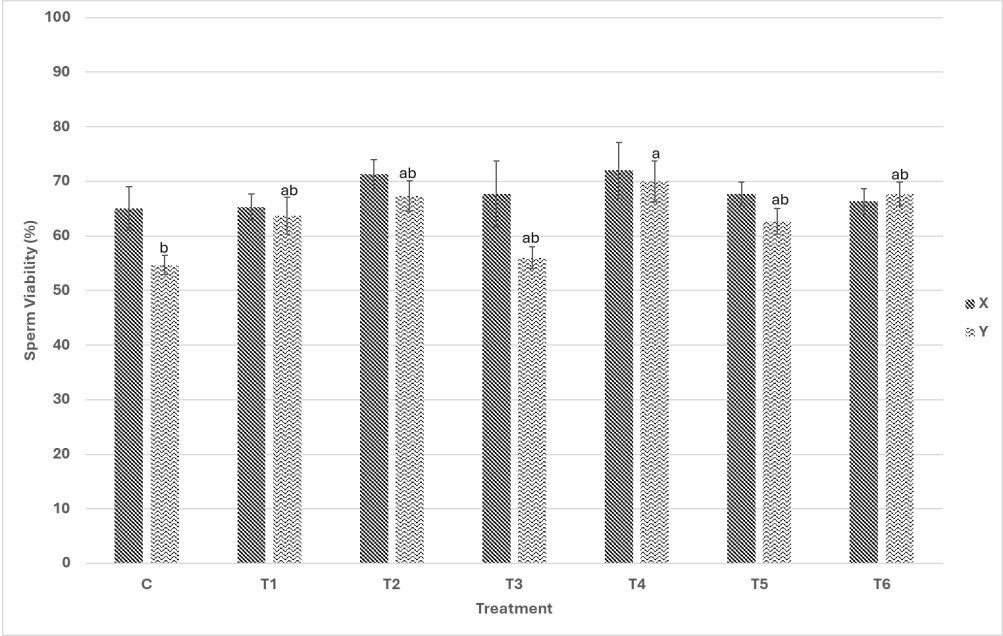
Motility		Treatment Group						
		C	T1	T2	T3	T4	T5	T6
X -bearing sperm								
Total Motility (%)		87.0± 1.5^{bc}	83.0±2.5^{c}	98.0±1.0^{a}	99.0±0.6^{a}	96.0±2.0^{ab}	92.0±2.0^{abc}	93.7±2.8^{b}
Progressive (%)		1.7±1.2^{d}	58.0±4.5^{b}	61.7±1.2^{b}	71.7±3.4^{ab}	76.7±0.9^{a}	32.7±6.2^{c}	21.0±1.5^{c}
Velocity Distribution (%)	Rapid	2.3±1.9^{b}	59.7±3.7^{a}	87.3±2.2^{a}	94.7±0.3^{a}	71.7±19.9^{a}	66.3±2.4^{a}	70.0±2.6^{a}
	Medium	84.7±1.7^{a}	23.3±1.2^{b}	11.0±1.5^{c}	4.3±0.3^{d}	3.7±0.9^{d}	26.0±0.6^{b}	23.3±1.3^{b}
	Slow	8.3±1.8^{a}	4.3±2.3^{ab}	1.3±0.3^{b}	0.7±0.3^{b}	0.7±0.^{3b}	2.0±0.0^{b}	3.0±0.0^{ab}
	Static	4.67±1.5^{a}	9.33±4.3	1±0.6	0.33±0.3	4±2.0	5.67±2.0	3.33±2.8
Y-bearing sperm								
Total Motility (%)		92.3±2.3^{a}	95.0±1.5^{a}	83.0±2.3^{ab}	83.7±4.6^{ab}	94.0±1.5^{a}	73.7±1.5^{b}	89.0±4.6^{a}
Progressive (%)		5.0±2.6^{c}	63.3±2.3^{a}	54.3±3.7^{a}	53.3±5.5^{a}	63.7±2.9^{a}	34.3±6.2^{b}	25.7±0.7^{b}
Velocity Distribution (%)	Rapid	11.3±5.2^{d}	72.7±3.5^{ab}	59.0±4.7^{bc}	54.7±5.0b^{c}	84.0±1.5^{a}	52.3±2.4^{c}	71.7±4.3^{abc}
	Medium	81.0±3.2^{a}	23.0±2.6^{bc}	24.0±4.5^{bc}	29.0±1.5^{b}	10.3±0.7^{c}	21.7±3.8^{bc}	17.3±2.8^{bc}
	Slow	6.7±1.9	1.7±0.3	3.0±1.2	6.7±1.9	3.7±1.5	3.0±0.6	3.0±0.6
	Static	1.3±0.3^{c}	3.3±1.5^{bc}	14.0±1.5^{ab}	10.7±3.8^{bc}	2.3±0.3^{c}	23.0±1.2^{c}	8.0±4.2^{bc}

Variables labelled with the same alphabet (a, b, c, d) indicate that their means are not statistically significantly different (P>0.05). Conversely, variables labelled with different alphabets are considered significantly different (P<0.05)

According to Table 4, sperm motility was highest in T3 (fresh young coconut water) and T4 (boiled young coconut water), with X-bearing sperm reaching nearly 100% motility (99.0±0.6% and 96.0±2.0%). This was a significant improvement compared to the control group (87±1.5%). The coconut water's antioxidants, sugars, and electrolytes appear to protect sperm cells from oxidative stress, which is essential for maintaining motility. Keeping such structures intact is critical, for oxidative stress is known to impair both vigor and motility through high concentrations of polyunsaturated fatty acids in sperm membranes, whose susceptibility towards oxidative degradation is high [27][24].

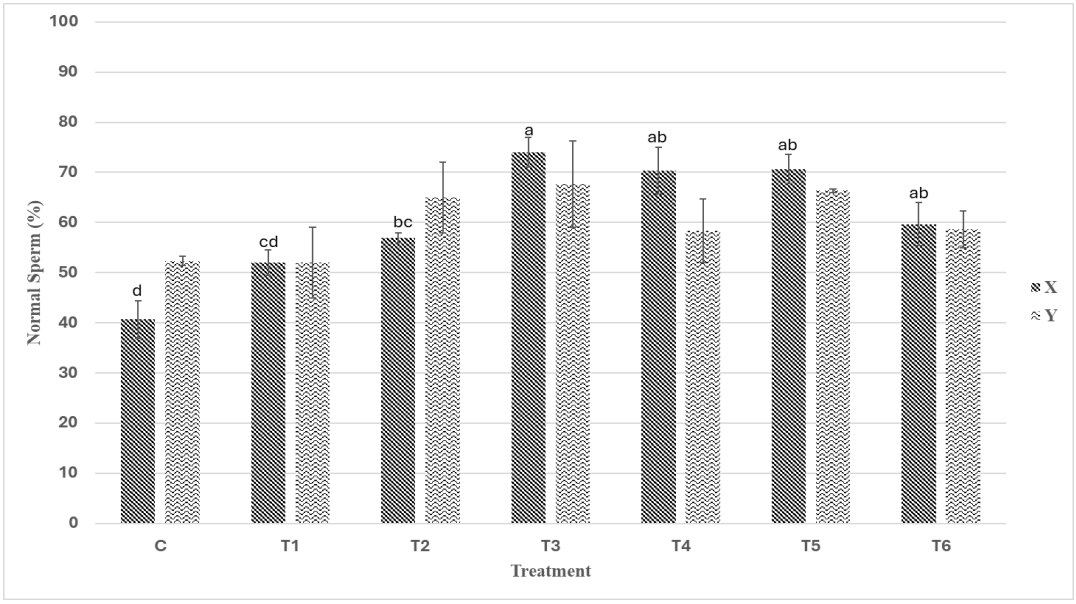
The boiling process in T4 further concentrated the beneficial compounds in coconut water, leading to higher sperm velocity. T4 exhibited most progressive motility, since concentrated antioxidants in boiling young coconut water can most effectively counteract ROS, defending motility best. Besides, fructose and glucose in coconut water maintain continuous energy for locomotory activity in sperm cells, supporting high velocity in extenders' treatments with coconut water [20]. Sustained motion through continuous delivery of such sugars helps in continuous motion of the flagellum, an integral part of sperm motility. In comparison, treatments using mature coconut water (T1 and T2) showed lower motility and velocity, suggesting that younger coconut water might be more effective in preserving sperm motility.
The control group, which used a tris-based solution, showed the lowest values in both motility and velocity, as it lacks the natural nutrients and antioxidants found in coconut water. This clearly shows that natural extenders, especially coconut water, have a greater impact on sperm motility and velocity than synthetic ones.

Viability and Morphology



Variables labelled with the same alphabet (a, b, c,d) indicate that their means are not statistically significantly different (P>0.05). Conversely, variables labelled with different alphabets are considered significantly different (P<0.05).

Figure 1. Sperm viability (mean±SEM) of X and Y sperm after EWS sperm sexing using different extenders*:* Control (C: tris based solution), Treatment 1 (T1: fresh mature coconut water), Treatment 2 (T2: boiled mature coconut water), Treatment 3 (T3: fresh young coconut water), Treatment 4 (T4: boiled young coconut water), Treatment 5 (T5: chicken egg yolk) and Treatment 6 (T6: duck egg yolk)



Variables labelled with the same alphabet (a, b, c,d) indicate that their means are not statistically significantly different (P>0.05). Conversely, variables labelled with different alphabets are considered significantly different (P<0.05).

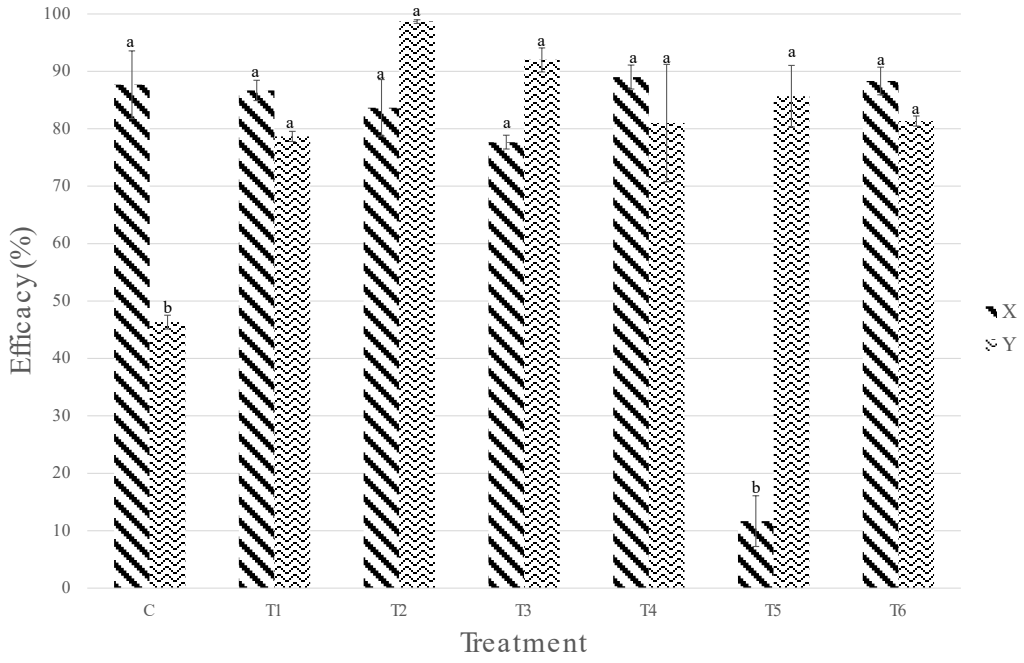
Figure 2. The percentage of normal sperm (mean±SEM) of X and Y sperm after EWS sperm sexing using different extenders: Control (C: tris based solution), Treatment 1 (T1: fresh mature coconut water), Treatment 2 (T2: boiled mature coconut water), Treatment 3 (T3: fresh young coconut water), Treatment 4 (T4: boiled young coconut water), Treatment 5 (T5: chicken egg yolk) and Treatment 6 (T6: duck egg yolk)

Figures 1 and 2 reveal that T4 (boiled young coconut water) provided the highest sperm viability for Y-bearing sperm (94.0%) and maintained better sperm morphology compared to the other treatments. Coconut water's antioxidants and electrolytes help maintain sperm structure and function, which is crucial for sperm viability. Boiling in T4 most likely maximizes availability of nutrition and antioxidants, and, subsequently, sperm cell viability and stability. Stability in such a form is significant in sustaining viability, for antioxidants in coconut water restrain oxidative stress-related cellular deterioration [7]. In addition, coconut water's electrolytes (sodium, potassium, and magnesium) preserve cellular pH and state of osmosis, both significant for viability in sperm [32][34]. Stable pH and balanced contribution of such compounds most likely counteract osmostatic shock and dehydration in cells, both of which can result in a viability drop in sperm.

In contrast, the tris-based solution (control) resulted in lower viability and a higher proportion of abnormal sperm, likely due to the lack of protective compounds. T3 (fresh young coconut water) also showed improved sperm viability, and a higher percentage of normal sperm compared to the control group. These results highlight the advantage of using natural extenders in improving sperm quality.

Regarding morphology, T3 (fresh young coconut water) had the highest proportion of normal X-bearing sperm (93.7%). This is likely because young coconut water contains essential amino acids and minerals, which contribute to maintaining sperm membrane integrity and reducing structural abnormalities. These findings are consistent with the idea that natural extenders help maintain sperm quality by providing the necessary nutrients. Sperm membrane integrity could possibly be sustained through such compounds, and thus, structural abnormalities could be fewer in such cases. In agreement with observation, tris-containing solutions, such as in controls, exhibited a relatively smaller proportion of healthy sperm. Sperm could become susceptible to membrane shifts and damage in tris-containing solutions, with no antioxidants and no naturally present nutrition, and thus, supporting the use of naturally rich extenders such as coconut water [11][2].

Separation Efficiency and Sperm Purity



Variables labelled with the same alphabet (a, b, c,d) indicate that their means are not statistically significantly different (P>0.05). Conversely, variables labelled with different alphabets are considered significantly different (P<0.05).

Figure 3. Efficacy of EWS separation method (mean±SEM) of X bearing and Y bearing sperm after sperm sexing using different extenders: Control (C: tris based solution), Treatment 1 (T1: fresh mature coconut water), Treatment 2 (T2: boiled mature coconut water), Treatment 3 (T3: fresh young coconut water), Treatment 4 (T4: boiled young coconut water), Treatment 5 (T5: chicken egg yolk) and Treatment 6 (T6: duck egg yolk).

Figure 3 The EWS method, as shown in Figure 3, was more efficient in producing high-purity X-bearing sperm when coconut water-based extenders were used. T3 (fresh young coconut water) and T4 (boiled young coconut water) achieved the highest purity levels (95.2% and 96.3%, respectively). This suggests that coconut water helps improve sperm buoyancy, facilitating better separation during the sperm sexing process. The unique composition of coconut water, with its high antioxidant and electrolyte content, likely aids in separating sperm based on their density, which is crucial for effective sperm sexing. Electrolytes such as magnesium, potassium, and calcium further enhance sperm viability by stabilizing cell membranes and maintaining the pH balance within the extender solution. A well-balanced electrolyte composition prevents harmful pH fluctuations that could compromise cellular function. The stable osmotic environment created by these minerals helps protect sperm from swelling or shrinkage, preserving their structural integrity [32][34].

On the other hand, the control group and treatments with mature coconut water (T1 and T2) had lower purity levels for X-bearing sperm, indicating that mature coconut water is less effective in improving sperm separation. The boiling process in T4, which concentrates beneficial compounds, also contributed to better sperm purity, reinforcing the positive impact of boiling on the efficacy of the extender.

Egg yolk (T6), particularly from duck eggs, also helped improve sperm purity due to the presence of phospholipids like lecithin and cholesterol, which stabilize sperm membranes during the separation process. This added stability is essential for maintaining sperm integrity and functionality during sperm sexing, as membrane damage can reduce sperm quality. Phospholipids like lecithin and cholesterol, which are critical for maintaining sperm membrane fluidity during temperature fluctuations. This property is especially valuable in the EWS process, as it minimizes the risk of membrane damage from thermal shifts. Lecithin and cholesterol act as membrane stabilizers, reducing the likelihood of cryoinjury by enhancing sperm cell resilience to temperature-induced stress [28][15]. This stabilizing effect ensures that sperm membranes remain intact and functional, promoting successful fertilization post-thaw.

Conclusions

In conclusion, this study highlights the significant role of natural extenders, such as young coconut water and egg yolk, in improving sperm viability, motility, and morphology, offering promising alternatives to traditional synthetic extenders for sperm sexing. Boiled young coconut water (T4) emerges as particularly effective, consistently enhancing both X- and Y-bearing sperm quality. These extenders provide a cost-effective and efficient method for sperm sexing, potentially making reproductive technologies more accessible to small-scale farmers and supporting genetic selection and sustainability in livestock breeding. Future research should focus on exploring the effects of varying incubation times and albumin concentrations to optimize sperm motility and viability post-sexing. Additionally, studies should include in vivo fertility trials to evaluate the success of artificial insemination (AI) using EWS-sorted sperm in Barbados Blackbelly ewes, as well as molecular techniques to accurately distinguish X and Y-bearing sperm.

Conflicts of Interest

We have no conflict of interest

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