Improvement of Sexed Sperm Quality of Bali Bulls by Adding Palmyra (*Borassus flabellifer Linn.*) Fruit Water to Citrate-Egg Yolk Extender

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ABSTRAK

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Sexing sperma merupakan metode yang dikembangkan untuk memisahkan sperma berkromosom X dan Y yang selanjutnya digunakan untuk produksi anak sesuai dengan jenis kelamin yang dinginkan peternak. Namun demikian, proses sexing menyebabkan penurunan kualitas sperma yang disebabkan oleh terjadinya stres oksidatif akibat peningkatan radikal bebas yang berlebihan. Dengan demikian, ke dalam bahan pengencer perlu menambahkan antioksidan dalam upaya untuk menangkal pengaruh negatif dari radikal bebas terhadap kehidupan sperma. Penelitian ini dirancang dengan menambahkan air buah lontar (PFw), yang kaya akan antioksidan ke dalam pengencer sitrat-kuning telur (CEy). Tujuan penelitian ini adalah untuk mengevaluasi potensi PFw sebagai suplemen antioksidan alami dalam pengencer CEy dalam upaya meningkatkan kualitas sperma sexing sapi Bali. Sperma ditampung dengan menggunakan metode vagina buatan dari tiga ekor sapi bali jantan berumur 3-4 tahun. Sperma yang berkualitas baik (motilitas ≥70%, abnormalitas ≤20%) di-sexing dengan metode gradien albumin tiga lapis (5, 10, dan 15 persen) selama 20 menit, dan sperma yang berada pada lapisan albumin terbawah dipreservasi dalam pengencer: CEy, PFw, PFw-kuning telur (PFw-Ey), atau CEy-PFw. Hasil penelitian menunjukkan bahwa preservasi sperma dalam pengencer CEy-PFw menghasilkan kualitas sperma yang lebih tinggi (P<0,05) dibandingkan dengan ketiga pengencer lainnya, kecuali pada parameter abnormalitas sperma (P>0,05). Disimpulkan bahwa penambahan PFw ke dalam pengencer CEy berpotensi meningkatkan kualitas sperma sexing sapi bali.

Kata Kunci: Sapi Bali, Sitrat, Kuning Telur, Air Buah Lontar, Sperma Sexing

ABSTRACT

Hine TM, Nalley WM, Marawali A, Kihe JN, Kune P, Uly K. 2024. Improve the sexed sperm quality of Bali bulls by adding palmyra (*Borassus flabellifer* Linn.) fruit water to citrate-egg yolk extender. JITV 29(3):135-142. DOI:http://dx.doi.org/10.14334/jitv.v28i3.3388.

Sperm sexing is a technique designed to separate sperm carrying the X and Y chromosomes, which are then used for artificial insemination to generate offspring with the sex the breeder desires. However, the sexing process causes a decrease in sperm quality caused by oxidative stress due to an excessive increase in free radicals. Thus, to counteract the detrimental effects of free radicals on sperm life, antioxidants must be added to the diluent. This study was designed by adding palmyra fruit water (PFw) to citrate-egg yolk (CEy) diluent. This study aimed to evaluate the potency of PFw as a natural antioxidant supplement in CEy diluent to improve the quality of sexing sperm in Bali bulls. Sperm were collected using the artificial vagina method from three Bali bulls aged 3-4 years. Good quality sperm (motility ≥70%, abnormality ≤20%) were sexed with a three-layer albumin gradient method (5, 10, and 15 percent) for 20 minutes, and sperm that were on the bottom albumin layer were preserved in CEy, PFw, PFw-egg yolk (PFw-Ey), or CEy-PFw. The results showed that sperm preservation in the CEy-PFw diluent resulted in higher sperm quality (P<0.05) compared to the other three diluents, except for the sperm abnormality parameter (P>0.05). It was concluded that adding PFw into the CEy diluent could potentially improve the sexed sperm quality of Bali bulls.

Key Words: Bali Bulls, Citrate, Egg Yolk, Palmyra Fruit Water, Sperm Sexing

INTRODUCTION

Sperm sexing is a method designed to improve the proportion of desired sex calves in calf populations (Seidel 2014; Razmkabir 2018; Vishwanath & Moreno 2018). In animals, such as cattle, there are two kinds of sperm: sperm with X and Y chromosomes. When the X sperm fertilizes the egg during fertilization, a female calf is born, while the Y sperm fertilization results in a male offspring. Naturally, a cattle 's ejaculate contains an equal amount of each type of sperm, 50 percent of which have the X chromosome and 50 percent the Y chromosome (Rai 2018). When a farmer wishes to grow male calves, sexing technology can reduce the quantity

of X sperm and raise the fraction of Y sperm, and vice versa (Kumar et al. 2016; Rai 2018; Yadav et al. 2018; Rahman & Pang 2020).

Sexing treatment causes a decrease in sperm fertility. According to Purwoistri et al.'s study, sperm motility decreased from 70 percent in fresh semen to 53.5 - 63.0 percent after sexing, sperm viability decreased from 95.12 to 91.91-93.30 percent, and sperm abnormalities rose from 5.28 to 6.82-8.76 percent (Purwoistri et al. 2013). The quality of frozen sexed-sperm was also lower compared to non-sexed-sperm after thawing, with a motility of 31.4 vs. 36.0 percent and viability 75.89 vs. 81.70 percent, respectively (Mahfud et al. 2019); the pregnancy rate dropped from 59.09 in nonsexed-sperm to 41.17 percent in sexed-sperm (Bhat & Sharma 2020). One reason for the decline in sperm fertility is the presence of oxidative stress, which is brought on by an excessive rise in free radicals during the sexing process. Antioxidants must be added to sperm diluent to neutralize these free radicals (Rath et al. 2013; Spinaci et al. 2016).

Previous studies have employed palmyra fruit water (PFw) to preserve the semen of Bali and Sumba Ongole bulls (Hine et al. 2014; Kaka et al. 2024). On the fourth day of preservation, the progressive motility of Bali bull sperm in the PFw-egg yolk diluent reached 44 percent, a significant increase over citrate-egg yolk's (CEy) 30 percent (Hine et al. 2014). More investigation by Kaka et al. (2024) revealed that using a nanoparticle diluent made from a mixture of 75% PFw and 25% egg yolk on Sumba Ongole bulls sperm can maintain progressive motility until the seventh day of preservation, with a percentage of progressive sperm motility reaching 40.20 percent. The outcomes were similar to 40.50 percent for the Cauda Epididymal Plasma-3 diluent.

Palmyra fruit has a high antioxidant content and radical scavenging activity (57.32–83.25%) (Wijewardana et al. 2016; Kurian et al. 2017). The phytosterols, flavonoids, saponins, triterpenoids, phenols, alkaloids, and tannins (Renuka et al. 2018; Huynh Thi Le et al. 2020), vitamin E (Huynh Thi Le et al. 2020), and β-carotene (Wijewardana et al. 2016) are a few of the antioxidants found in palmyra fruit. The palmyra fruit extract contained a total of 104 g GAE/100 mg and 98.40 g QE/100 mg phenolics and flavonoids, respectively, vitamin E (52.15-55.12 mg/100 g) and β-carotene (617.55 -2647.19 µg/100 g) (Wijewardana et al. 2016; Huynh Thi Le et al. 2020). The range of 2.2-difenill-1-pikrilhidrazil (DPPH) radical inhibition was between 35 to 70 percent; the 3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) radicals' inhibition ranged from 40 to 75.5 percent. Palmyra fruit extract has a nitric oxide scavenging activity of 45 to 76 percent and a superoxide radical scavenging activity of 43 to 83 percent (Renuka et al., 2018). Additionally, the palmyra fruit has small amounts of fat (3.64–3.95%) and protein (3.50–4.09%) (Wijewardana et al. 2016), potassium (237.00-276.73 mg/100g), magnesium (211.61-293.62 mg/100g) and saponins (36.10–55.62 g/100g) (Abe-Inge et al. 2018).

This study aimed to evaluate the potency of PFw as a natural antioxidant supplement in CEy diluent to improve the quality of sexing sperm in Bali bulls.

MATERIALS AND METHODS

Animals

Three Bali bulls aged 3-4 years old kept in the Williams & Laura Foundation experimental pen, Kupang Tengah District, Kupang Regency, Indonesia, were used for semen collection. The bull received up to 10% of its weight in forage, 3 kg of concentrate daily, and unlimited water access.

Diluent preparation

There were four diluents prepared in this study, namely citrate-egg yolk (CEy), palmyra fruit water (PFw), palmyra fruit water-egg yolk (PFw-Ey), and citrate-egg yolk-palmyra fruit water (CEy-PFw) (Table 1). Citrate diluent was prepared as described by (Arifiantini et al. 2010), namely 2.9 g of sodium citrate dehydrate (Sigma Aldrich, W302600-SAMPLE-K) dissolved in 100 mL of aquabidest until homogeneous.

Table 1. Composition of diluent

Ingredient	Diluent			
	CEy	PFw	PFw-Ey	CEy-PFw
Sodium citrate dehydrate (g)	2.9	-	-	2.9
Aquades (mL)	100	-	-	50
Egg yolk (mL)	20	-	20	20
PFw	-	100	100	50
Penicillin G	1000	1000	1000	1000
Streptomycin sulfate (µg)	1000	1000	1000	1000

CEy= citrate-egg yolk, PFw= palmyra fruit water, PFw-Ey= palmyra fruit water-egg yolk, CEy-PFw= citrate-egg yolk-palmyra fruit water

Palmyra fruit water is taken from young palmyra fruits: the tip of a young palmyra fruit was cut with a sterile knife, and water was drawn into the Erlenmeyer tube using a 20 mL syringe (Hine et al. 2014). For each diluent, 20% egg yolk (v/v), 1000 IU penicillin G sodium salt (Sigma Aldrich, P3032-10MU), and 1000 µg Streptomycin Sulfate Salt (Sigma Aldrich, S1277-5G) per mL.

Semen collection and evaluation

Semen was collected utilizing the artificial vaginal technique (Sylla et al. 2015; Setiadi et al. 2022). Semen was assessed macroscopically for color, volume, consistency, and pH. Under a microscope (Zeiss) with a magnification range of 100 to 400x, sperm were evaluated microscopically for mass movement and percentage of progressive motility (0= non-motile, 100= 100% progressive motility); eosin-negrosin staining was used to determine the viability of the sperm (Haris et al. 2020), sperm concentration asses using a Neubauer hemocytometer counting chamber (Crespilho et al. 2017) and sperm morphology was measured by carbolfuchsin-eosin (Morrell et al. 2018).

Sexing and sperm preservation

An albumin gradient of 5%, 10%, and 15% is used in the sperm sexing method, as carried out by Ratnawati et al. (2020) with slight modifications. The four diluents (CEy, PFw, PFw-Ey, CEy-PFw) were utilized to prepare the three albumin gradients. Approximately 2 mL of a 5-10-15 percent albumin gradient should be placed in a test tube, and fill the test tube to just over the 5 percent albumin gradient with 2 mL of diluted semen (1:1), incubate for 20 minutes until the albumin gradient layer was formed. Put 2 mL of the albumin gradient at the bottom of the tube into a test tube with 3 mL of diluent (CEy, PFw, PFw-Ey, CEy-PFw), centrifuge for five minutes at 1500 rpm, remove 3 mL of the supernatant; after that, the final 2 mL of precipitate were diluted using one of the four diluents (1: 2) and kept at 3 to 8 ºC in the refrigerator.

Assessing the quality of sexed sperm

During the 96-hour storage period, the quality of the sexed sperm was assessed every 24 hours. Sperm motility was assessed by applying a 10 µL drop of diluted semen to a warm slide with a glass coverslip and monitored in five microscopic fields of view at 400X magnification. The motility score was computed from the average movement of the five visual fields. Sperm viability was assessed by eosin-nigrosin staining (Haris et al. 2020). 10 µL drop of diluted semen on a slide and 40 µl drop of nigrosin–eosin; it smears on a slide and dries quickly in the heating stage (80ºC). Observe 200 sperm cells in a total of 10 tiny fields of view. Sperm were classified as viable (unstained) or dead (stained). Sperm morphology was assessed using the same method as the viability evaluation procedure; however, the proportion of sperm with aberrant morphology was measured. The integrity of the acrosome was assessed by diluting 500 µl semen combined with 50 µl of 1% formaldehyde citrate in a test tube (Mughal et al. 2013). A phase-contrast microscope at 1000X examined the sperm (200 in total) for their usual apical ridge. Evaluating the integrity of the plasma membrane using the hypoosmotic swelling (HOS) test as described by (Ramu and Jeyendran 2013; Zubair et al. 2013). 200 sperm were counted for swelling/coiling of the tail under a 400X phase-contrast microscope observation.

Designing experiments and data analysis

This study, with four treatments and five replicates for 20 trial units, was built using a completely randomized design. Data were analyzed using ANOVA, followed by Duncan's test. Analysis was performed using SPSS 25 software.

RESULTS AND DISCUSSION

Motility, viability, and abnormalities of sexed sperm

After dilution, sperm motility and viability decreased slightly; however, sperm abnormalities increased in all treatments compared to fresh semen; this could be due to sperm stress during the sexing process, which lowers sperm quality. Nonetheless, there was no significant difference between the treatments ($P>0.05$) (Table 2).

Compared to PFw and PFw-Ey, CEy-PFw generated more viable and motile sperm from the first to the fourth preservation day ($P<0.05$). On the other hand, sperm motility and viability showed significant differences with CEy ($P<0.05$) only on the third and fourth days of preservation, respectively. On the fourth day of preservation, the sperm motility and viability in CEy-PFw diluent were noticeably higher than in CEy diluent (43.3 and 51.0 vs. 35.0 and 41.3%) (Table 2); this indicates that sperm motility and viability were improved by adding palmyra fruit water to the citrate-egg yolk diluent; this is possible due to the presence of several compounds in Palmyra fruit water that are highly advantageous for sperm life during preservation.

The reason for the low sperm motility and viability in PFw and PFw-Ey diluents is that they lack buffer elements, which help to keep the diluent's pH within the ideal range for sperm life, as well as protective elements

against cold shock in the form of lipoproteins and lecithin found in egg yolks. When measured on the fourth day of preservation, the pH of the PFw and PFw-Ey diluents dropped to 6.2, but the pH of the CEy and CEy-PFw diluents ranged from 6.4 to 6.7 (data not shown). Compared to the ideal sperm diluent pH of 6.8 to 7.2, the pH of PFw and PFw-Ey diluents is significantly lower (Liu et al. 2016). The proportion of abnormal sperm in fresh semen was 3.92%, which is still below the threshold of 20% for artificial insemination. Sperm abnormalities increased to 4.50–5.14% in all four diluents after being preserved for 4 days. From the post-dilution to the fourth day of storage, there was no discernible variation in the percentage of abnormal sperm across diluents ($P>0.05$); this implies that the variations in nutrient content among diluents have no effect on the percentage of abnormal sperm.

Sperm dilution is one method used to ensure sperm survival in an in-vitro environment. Dilution allows for maintaining the extender's pH and sperm metabolism; bacterial contamination is minimized, and cryogenic damage during preservation and cryopreservation can be suppressed (Malik et al. 2018; Raheja et al. 2018). A good sperm diluent should be able to keep the pH between 6.8 and 7.2 (Liu et al. 2016), provide energy (Mohamed et al. 2019), contain antioxidants to lessen stress brought on by free radicals (Mousavi et al. 2019), contain antibiotics to prevent contamination (Schulze et al. 2020), and act as an anti-cold shock (Amirat-Briand et al. 2010; Tariq et al. 2020). Diluents with these qualities can protect sperm life throughout storage and transportation, enabling their use in artificial insemination. Sperm extenders consist of two separate types: liquid and frozen. While frozen sperm can survive for years, liquid sperm can only last a few days (Johnston et al. 2012). Some scientists have created sperm diluents using a variety of components such as palmyra juice (Hine et al. 2014) and egg yolk (Filho et al. 2009), which are both sourced from plants and animals, which can sustain sperm quality, are readily available and inexpensive priced (Hine et al. 2014; Layek et al. 2016).

This study supports using PFw as a supplemental ingredient in the CEy extender. Adding PFw to the diluent will create a more favorable in vitro environment for sperm life; this is strongly tied to the existence of critical substances in PFw, including numerous antioxidants that help fight free radicals and carbohydrates that may serve as the sperm's potential energy source (Renuka et al. 2018; Behera & Nayak, 2022).

Palmyra fruit water has a high carbohydrate content of 10.96 percent (Haisya et al. 2011), which can be converted into glucose and fructose, two crucial energy sources for sperm movement and viability (Mukai & Okuno 2004). A study by Arifiantini and Purwantara (2010) demonstrated that sperm motility was improved when fructose was added to the citrate-yolk diluent. Since fructose and glucose are simple sugars with a low molecular weight, they may easily pass through cell membranes. Through the activity of an enzyme found within the cell, glucose and fructose may be metabolized into energy through the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Ford 2006; Yi et al. 2008; du Plessis et al. 2015). The energy generated is subsequently used by sperm to sustain their viability and motility during preservation. ATP can be regenerated by adding a phosphoryl group from the extenders' carbohydrates or lipids. Thus, the quantity of energy produced for sperm life will depend on the amount of carbohydrates in the extender. According to Tourmente et al. (2015), the ATP compound in bovine sperm is closely related to sperm.

Palmyra fruit water also contains high levels of flavonoids and phenolics (Saranya and Vijayakumar 2016), which act as antioxidants to protect sperm against free radical attacks. Flavones and catechins are the most potent flavonoids that protect cells against reactive oxygen species (Panche et al. 2016) produced during metabolic processes. Reactive oxygen species cause damage to cell membranes, which is caused by lipid peroxidation. Damage to the cell membrane causes cell charge modification, changes in osmotic pressure, cell swelling, and death. Flavonoids can neutralize free radicals in various ways, such as by oxidizing them to create other harmless radicals, or they may scavenge superoxide and peroxynitrite directly, two radicals of highly reactive oxygen.

However, using Palmyra fruit water alone has not adequately preserved sperm survival during in vitro preservation, suggesting that sperm require other nutrients besides carbohydrates, which are available in Palmyra fruit water in sufficient concentrations. When combined with egg yolk in a citrate diluent, palmyra fruit water can enhance sperm quality more effectively than palmyra fruit water alone; i.e., motility, viability, plasma membrane integrity, and acrosomal integrity all rose by 38.33; 41,93; 42,32; 42,54 percent, respectively, which were observed on the fourth day of storage, suggesting that sperm requires the inclusion of egg yolk in the diluent.

Previous studies have listed several substances in egg yolks, including phospholipid, cholesterol, and low-density lipoprotein (LDL) (Layek et al. 2016; Anzar et al. 2019; Sun et al. 2020). Low-density lipoproteins work by stabilizing the sperm membrane and replacing phosphoproteins in the sperm membrane that have been disturbed during preservation, increasing cold tolerance to protect sperm from being damaged by cold shock, and increasing the cholesterol/phospholipid ratio to prevent the phospholipids in membranes from degrading (Lagace & Ridgway 2013; Stevenson et al. 2014). LDL-derived lipid-binding proteins in the yolk

Table 2. Viability, motility, and abnormality of sexed sperm of Bali bulls in four different extenders

Parameters	Treatments	Fresh semen	Post-dilution (Day 0)	Day 1	Day 2	Day 3	Day 4
Sperm motility(%)	CEy	79.5±4.32^{a}	77.5±2.74^{a}	67.5±2.74^{a}	57.5±2.74^{a}	45.0±3.16^{b}	35.0±4.47^{b}
	PFw	79.5±.4.32^{a}	77.5±2.74^{a}	28.3±4.08^{c}	7.5±2.74^{c}	5.0±0.00^{d}	5.00±0.00^{d}
	PFw-Ey	79.5±4.32^{a}	77.5±2.74^{a}	60.8±4.92^{b}	45.0±8.37^{b}	25.0±11.4^{c}	15.8±12.4^{c}
	CEy-PFw	79.5±4.32^{a}	77.5±2.74^{a}	69.5±3.94^{a}	60.7±3.56^{a}	53.3±2.58^{a}	43.3±2.58^{a}
	p-value	1.00	1.00	0.00	0.00	0.00	0.00
Sperm viability (%)	CEy	84.1±2.66^{a}	83.1±1.78^{a}	74.0±2.67^{a}	64.8±3.26^{a}	52.9±2.40^{a}	41.3±3.53^{b}
	PFw	84.1±2.66^{a}	82.2±2.06^{a}	35.1±5.31^{c}	15.8±1.93^{c}	11.9±1.82^{c}	9.09±0.56^{d}
	PFw-Ey	84.1±2.66^{a}	82.8±1.42^{a}	67.1±3.73^{b}	51.6±6.57^{b}	35.6±9.81^{b}	22.4±12.3^{c}
	CEy-PFw	84.1±2.66^{a}	83.4±1.58^{a}	77.0±3.32^{a}	67.6±4.12^{a}	57.8±5.42^{a}	51.0±2.12^{a}
	p-value	1.00	0.68	0.00	0.00	0.00	0.00
Sperm abnormalities (%)	CEy	3.92±0.35^{a}	4.06±0.78^{a}	3.94±1.01^{a}	4.17±0.61^{a}	4.46±0.69^{a}	4.50±0.51^{a}
	PFw	3.92±0.35^{a}	4.48±0.72^{a}	4.24±0.79^{a}	4.66±1.04^{a}	4.62±1.24^{a}	5.14±1.43^{a}
	PFw-Ey	3.92±0.35^{a}	4.16±0.43^{a}	4.13±0.61^{a}	4.31±0.99^{a}	4.61±1.00^{a}	4.67±0.61^{a}
	CEy-PFw	3.92±0.35^{a}	4.09±0.92^{a}	4.28±0.59^{a}	4.48±0.77^{a}	4.49±1.00^{a}	4.58±0.86^{a}
	p-value	1.00	0.75	0.87	0.79	0.99	0.63

CEy= citrate-egg yolk, PFw= palmyra fruit water, PFw-Ey= palmyra fruit water-egg yolk, CEy-PFw= citrate-egg yolk-palmyra fruit water. Different superscripts in the same column differ significantly (P<0.05)

Table 3. Plasma membrane and acrosome integrity of sexed sperm of Bali bulls in four different extenders

Parameters	Treatments	Fresh semen	Post-dilution (Day 0)	Day 1	Day 2	Day 3	Day 4
Plasma membrane integrity of sperm (%)	CEy	86.2±1.24^{a}	85.5±1.98^{a}	76.5±3.35^{a}	67.4±3.25^{a}	55.3±3.31^{a}	43.5±3.48^{b}
	PFw	86.2±1.24^{a}	84.3±0.20^{a}	39.4±5.83^{c}	20.2±6.51^{c}	14.9±2.19^{c}	11.2±0.92^{d}
	PFw-Ey	86.2±1.24^{a}	85.4±2.04^{a}	69.2±3.69^{b}	53.8±6.99^{b}	37.6±9.54^{b}	24.4±12.2^{c}
	CEy-PFw	86.2±1.24^{a}	85.7±1.95^{a}	79.3±3.32^{a}	70.2±4.92^{a}	60.1±6.22^{a}	53.5±3.01^{a}
	p-value	1.00	0.64	0.00	0.00	0.00	0.00
Acrosomal integrity of sperm (%)	CEy	87.4±0.66^{a}	86.2±1.72^{a}	76.9±3.28^{a}	67.7±3.87^{a}	55.9±3.31^{a}	44.4±3.62^{b}
	PFw	87.4±0.66^{a}	85.2±2.19^{a}	41.0±5.75^{c}	21.0±6.62^{c}	15.6±2.28^{c}	11.6±0.58^{d}
	PFw-Ey	87.4±0.66^{a}	85.6±2.34^{a}	70.1±3.91^{b}	54.6±6.65^{b}	38.5±9.58^{b}	25.1±12.1^{c}
	CEy-PFw	87.4±0.66^{a}	86.0±2.38^{a}	80.1±3.20^{a}	71.1±4.68^{a}	61.0±6.03^{a}	54.1±2.43^{a}
	p-value	1.00	0.87	0.00	0.00	0.00	0.00

CEy= citrate-egg yolk, PFw= palmyra fruit water, PFw-Ey= palmyra fruit water-egg yolk, CEy-PFw= citrate-egg yolk-palmyra fruit water. Different superscripts in the same column differ significantly (P<0.05)

protect sperm during freezing (Tarig et al. 2017; Raheja et al. 2018). The sperm protection mechanisms of LDL are as follows: 1). The plasma membrane of the sperm is attached to LDL phospholipids, which stabilize the membrane (Naz et al. 2018); 2) egg yolk phospholipids replace damaged plasma membrane phospholipids during sperm preservation and cryopreservation (Layek et al. 2016); and 3) seminal plasma protein interacts with LDL plasma protein (Manjunath 2018), which is responsible for the degradation of phospholipids and sperm cholesterol. Phospholipididylcholine (lecithin), the primary phospholipid in egg yolks, and lipoproteins help protect sperm against cold shock (Alvarez-Rodríguez et al. 2013). When sperm are cooled and frozen, the cholesterol-to-phospholipid ratio in the sperm membrane is disrupted mainly through the excretion of

cholesterol and the production of various reactive oxygen species (Raheja et al. 2018).

Plasma membrane and acrosome integrity of sexed sperm

Following dilution, the integrity of the plasma membrane and acrosome revealed no discernible variation ($P > 0.05$) between the treatments; this is due to the sperm not having gone through the cooling process, which could have harmed the sperm membrane. Lipid peroxidation induces modifications in the membrane structure of sperm during the cooling process from room temperature to 5 C. The reduction in sperm plasma membrane and acrosome integrity across all diluents indicates this. The integrity of the acrosome dropped from 87.4 to 11.6-54.1 percent on the fourth day of preservation, and the plasma membrane integrity dropped from 86.3 percent in fresh semen to 11.2-53.5 percent (Table 3).

CEy-PFw produced the highest levels of plasma membrane and acrosome integrity compared to the other three treatments, and from the first to the fourth day of preservation, it differed significantly from PFw and PFw-Ey ($P<0.05$). Only on the fourth day of preservation were discernible differences with CEy ($P<0.05$); this demonstrates that combining CEy with palmyra fruit water can result in a more optimal medium condition for preserving the integrity of the sperm's plasma membrane and acrosome during preservation.

Palmyra fruit water contains high levels of flavonoids and phenolics (Saranya and Vijayakumar 2016), which act as antioxidants to protect sperm membranes against free radical attacks. Flavones and catechins are the most potent flavonoids that protect cells against reactive oxygen species (Panche et al. 2016) produced during metabolic processes. Reactive oxygen species cause damage to cell membranes, which is caused by lipid peroxidation. Damage to the cell membrane causes cell charge modification, changes in osmotic pressure, cell swelling, and death. Flavonoids can neutralize free radicals in various ways, such as by oxidizing them to create other harmless radicals, or they may scavenge superoxide and peroxynitrite directly, two radicals of highly reactive oxygen.

This research confirms the effectiveness of palmyra fruit water as a component of citrate-egg yolk extender for sperm preservation in Bali bulls' sexed sperm. However, more research is needed on some sperm fertility issues when used for artificial insemination.

CONCLUSION

Palmyra fruit water has the potential to be a natural antioxidant supplement in citrate-egg yolk diluent to improve the sexed sperm quality of Bali bulls.

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