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THE EFFECTIVENESS OF VITAMIN C AND E ADDITION IN VARIOUS DILUENTS OF BRAHMAN CATTLE SPERMATOZOA AFTER FREEZING

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ABSTRACT

This research aimed to determine the quality of Brahman cattle spermatozoa after freezing in various diluent ingredients (Egg Yolk Citrate, Egg Yolk Tris, Skim Milk) which added vitamin C and vitamin E. The research was carried out using 4 treatments with 3 different diluent ingredients, each of which was Vitamins C and E are added to each diluent ingredient at a dose adjusted to the type of diluent agent. The treatment given is P0: Diluent without Vitamin C and E; P1: Diluent + Vitamin C; P2: Diluent + Vitamin E; and P3: Diluent + Vitamin C and Vitamin E. Research data were analyzed using analysis of variance and followed by Duncan's test at the 5% and/or 1% level for different variables. The results showed that the addition of vitamin C and vitamin E had a significant effect (P<0.05) on motility and viability in the egg yolk tris diluent and egg yolk citrate but had no significant effect (P>0.05) in the skim milk diluent. The use of different diluents had no significant effect (P>0.05) on motility, viability, and abnormalities. This research concludes that Vitamin C added to the diluent tris egg yolk, egg yolk citrate, and skim milk showed higher average motility, percentage of live spermatozoa, and abnormalities of post-freezing Brahman cow spermatozoa.

Key words: Brahman cattle, diluent ingredients, spermatozoa quality, vitamin C, vitamin E

ABSTRAK

Penelitian ini bertujuan mengetahui kualitas spermatozoa sapi Brahman pasca pembekuan pada berbagai bahan pengencer (Sitrat Kuning Telur, Tris Kuning Telur, Susu Skim) yang ditambahkan vitamin C dan vitamin E. Penelitian dilaksanakan dengan menggunakan empat perlakuan pada tiga bahan pengencer yang berbeda yang masing-masing bahan pengencer ditambahkan vitamin C dan E dengan dosis yang disesuaikan dengan jenis bahan pengencernya yakni bahan pengencer tanpa vitamin C dan E (P0); bahan pengencer + vitamin C (P1); Bahan pengencer + vitamin E (P2); dan bahan pengencer + vitamin C dan vitamin E (P3). Data hasil penelitian dianalisis menggunakan analisis ragam dan dilanjutkan dengan uji Duncan. Hasil penelitian menunjukkan bahwa penambahan vitamin C dan vitamin E berpengaruh nyata (P<0,05) terhadap motilitas dan viabilitas pada pengencer tris kuning telur dan sitrat kuning telur namun tidak berpengaruh nyata (P>0,05) pada pengencer susu skim. Penggunaan bahan pengencer yang berbeda tidak berpengaruh nyata (P>0,05) terhadap motilitas, viabilitas dan abnormalitas. Kesimpulan dari penelitian ini adalah vitamin C yang ditambahkan pada bahan pengencer Tris Kuning Telur, Sitrat kuning Telur dan Susu Skim menunjukkan peningkatan motilitas, persentase spermatozoa hidup, dan penurunan abnormalitas spermatozoa sapi Brahman.

Kata kunci: Sapi Brahman, bahan pengencer, kualitas spermatozoa, vitamin C, vitamin E

INTRODUCTION

The success of artificial insemination (AI) technology is influenced by the quality of frozen sperm, making it essential to maintain the quality of frozen semen. One approach to preserving the quality of frozen sperm is by adding an extender, which is necessary to sustain sperm viability during freezing, storage, and after thawing before it is used for insemination.

Some of the extenders used for producing frozen semen include Citrate Egg Yolk (CEY), Tris Egg Yolk (TEY), and Skim Milk (SM). However, during the freezing and storage process, a decline in quality and sperm mortality occurs due to lipid peroxidation by free radicals, leading to a decrease in the quality of frozen semen. To combat sperm mortality caused by free radicals, antioxidants must be added to the extender (Blegur *et al*. 2020). Antioxidants are substances that can neutralize free radicals or act as agents that prevent biological systems from the harmful effects arising from processes or reactions that cause excessive oxidation (Ames *et al.* 1993; Almey *et al*. 2010). According to Sukmawati *et al*. (2014), lipid peroxidation causes damage to the sperm plasma membrane. The damage caused by free radicals and lipid peroxidation can reduce the motility and viability of sperm (Sartika *et al*. 2022). Vitamin C and E are substances capable of scavenging free radicals, thus maintaining sperm quality during the freezing, storage, and thawing processes.

Vitamin C has the ability to strengthen the stability of the protective tissue of the plasma membrane against lipid peroxidation, thereby preserving semen quality and fertility. Suryohudoyo (2000) stated that vitamin C is an antioxidant capable of breaking the chain reaction of free radicals. The research of Savitri *et al*. (2014) showed that the administration of 3.5 mM vitamin C resulted in higher post-freezing sperm quality compared to 1.5 mM and 2.5 mM. Vitamin E can transfer phenolic hydrogen to free radicals from polyunsaturated fatty acids that have undergone peroxidation, thereby breaking various free radical chain reactions. Alawiyah and Hartono (2006) reported that the addition of vitamin E in the CEY extender for freezing goat sperm produced the best results at 0.4 g/100 mL of extender compared to smaller doses.

MATERIALS AND METHODS

Implementation of Research

This study was conducted at the Laboratory of the Regional Technical Service Unit (UPTD) of the Artificial Insemination Center (BIB) in Terbanggi Besar Subdistrict, Central Lampung Regency, Lampung Province. The research design used was a Completely

Randomized Design (CRD) with 4 treatments and 6 replications. The study was carried out using four treatments across three different extenders, with each extender supplemented with vitamin C and E at doses adjusted according to the type of extender: extender without vitamin C and E (P0); extender + vitamin C (P1); extender + vitamin E (P2); and extender + vitamin C and E (P3). The extenders used were TEY, CEY, and SM, with compositions as shown in Table 1. The semen used was fresh semen from Brahman cattle.

The procedure for preparing the extenders followed the guidelines of BIB Poncowati (2012) and BIB Lembang (2009). The process of semen collection was based on the guidelines of Toelihere (1993), while the stages of frozen semen preparation followed BIB Poncowati (2012).

Measured Parameters

The measured parameters included motility (movement), viability (percentage of live sperm), and abnormalities.

Data Analysis

The research data were analyzed using analysis of variance (ANOVA) and followed by Duncan's test.

RESULTS AND DISCUSSION

The results of the study on the addition of vitamin C and E to Tris TEY, CEY, and SM extenders on the motility of Brahman cattle spermatozoa after freezing are presented in Table 2 and Figure 1. The analysis of variance showed that the addition of vitamins C and E had a significant effect ($P<0.05$) on sperm motility after freezing in TEYand CEY extenders, but did not show a significant difference in the skim milk extender. However, there were no significant differences ($P>0.05$) in motility among the different extenders used. This is because all three extenders contain substances capable of maintaining sperm motility and viability. The average percentage of motility of Brahman cattle sperm after freezing and thawing indicated that the addition of vitamin C resulted in the highest post-thaw motility across all three extenders.

Figure 1 shows that, across all extenders, the addition of a combination of vitamin C and vitamin E (P3) resulted in the lowest values compared to P0, P1, and P2. This is suspected to be due to the use of excessive doses of antioxidants, which can affect the rate of oxidation. Excessive antioxidants can have a negative impact on sperm cells (Abdillah 2018). This is supported by Savitri *et al*. (2014), who state that excessive doses of antioxidants can affect the oxidation rate, leading to the depletion of antioxidant activity; in fact, excess antioxidants can become pro-oxidants. Excessive antioxidants can result in a medium viscosity, which negatively affects spermatozoa. As viscosity increases, the higher the viscosity, the more it inhibits motility. Excessive antioxidant doses can cause the solution to become hypertonic. Hypertonic extenders can damage the plasma membrane and hinder spermatozoa metabolism, reducing the energy required for movement and thereby decreasing motility (Hartono 2008).

The addition of vitamin C (P1) to TEY, CEY, and SM extenders resulted in the highest values. This is likely because vitamin C can provide protection to the sperm's plasma membrane during freezing. Azawi and

Table 1. Diluent composition

Ingredients	Tris egg yolk	Citrate egg yolk	Skim milk
Tris amino methane (g)	3.03	-	-
Citric acid (g)	1.78	2.8	-
Fructose (g)	1.25	-	-
Glukosa (g)	-	2.85	-
Skim milk (g)	-	-	10
Aquadest (mL)	100	100	96
Egg yolk (mL)	20	20	20
Streptomycin (g)	3	3	3
Penicillin (g)	1	1	1
Gliserol (mL)	6	6	6
Vitamin C (g)/100 mL	0.2	0.5	0.25
Vitamin E (g)/100 mL	0.41	0.41	0,41

Table 2. Spermatozoa quality of Brahman cow spermatozoa after freezing

Variable	Treatment	Extenders			
		P0	P1	P2	P3
Motilitas	TEY	44.33±2.50^{b}	46.16±1.47^{b}	44.83±2.92^{b}	41.50±1.37^{a}
	CEY	45.00±1.26^{b}	48.50±0.84^{c}	41.50±1.76^{a}	40.17±1.33^{a}
	SM	43.83±8.75	47.16±6.27	45.66±7.08	42.66±7.91
Viability	TEY	44.33±2.50^{b}	46.16±1.47^{b}	44.83±2.92^{b}	41.50±1.37^{a}
	CEY	45.00±1.26^{b}	48.50±0.84^{c}	41.50±1.76^{a}	40.17±1.33^{a}
	SM	43.83±8.75	47.16±6.27	45.66±7.08	42.66±7.91
Abnormality	TEY	8.57±2.06	8.33±1.47	8.25±2.75	9.13±2.03
	CEY	9.29±1.53	10.40±1.437	10.32±1.12	9.68±1.90
	SM	9.36±2.08	8.73±1.87	8,87±2,20	9.68±2.06

^{a,b}Difference superscripts on the same row indicate significant differences ($P<0.05$), TEY= Tris egg yolk, CEY= Citrate egg yolk, SM= Skim milk, P0 =No vitamin added, P1= Addition of vitamin C; P2= Addition of vitamin E, P3= Addition of a combination of vitamin C and vitamin E

Hussein (2013) reported that vitamin C can preserve spermatozoa during dilution, cooling, and storage. Vitamin C also enhances the stability of free radicals that affect spermatozoa (Praditasari 2017). Aslam *et al*. (2014) stated that the addition of vitamin C to extenders optimizes the rate of fructolysis, thus meeting the energy needs for sperm movement and survival. Additionally, vitamin C can scavenge oxygen radicals within cells, preventing lipid peroxidation that inhibits glycolysis, and motility. Vitamin C strengthens the stability of the protective tissue of the plasma membrane against lipid peroxides, thus preserving the quality and fertility of spermatozoa (Savitri *et al*. 2014).

The addition of vitamin E to the extenders did not show a significant difference compared to P0. This is likely because vitamin E may not have been fully dissolved, reducing its effectiveness as an antioxidant. Hashem *et al*. (2017) noted that in semen extenders, vitamin E should be dissolved with Tween 80, which contains oleic acid, to improve sperm quality. In this study, Tween 80 was not used as a solvent, resulting in less optimal outcomes due to undissolved particles. The motility of individual Brahman cattle spermatozoa in frozen semen added with vitamin C and E in skim milk extenders showed no significant difference (P>0.05). This may be due to both vitamins C and E contain antioxidants that neutralize free radicals and help maintain frozen semen motility. Yahaq (2019) states that the appropriate dose of vitamin C in extenders can improve semen quality, particularly motility, as vitamin C antioxidants can prevent lipid peroxidation in sperm cell membranes by quenching or reducing free radicals and ending the reaction cycle. Beconi *et al*. (1993) state that vitamin E acts as an antioxidant to inhibit lipid peroxidation caused by free radicals. The assessment of Post Thawing Motility (PTM) resulted in a motility percentage above 42.66%, indicating that the semen can still be distributed to inseminators for artificial insemination. According to the BSN (2017), semen ready for distribution should have an individual motility of ≥ 40%.

The viability or percentage of live Brahman cattle spermatozoa after freezing with various extenders containing vitamin C and E is presented in Table 2 and Figure 2. The analysis of variance showed that the addition of vitamins C and E had a significant effect (P<0.05) on spermatozoa viability after freezing in the TEY extender, but no significant difference was observed in the CEY and SM extenders. The use of different extenders (TEY, CEY, and SM) did not show a significant difference (P>0.05) in the viability of Brahman cattle spermatozoa after freezing.

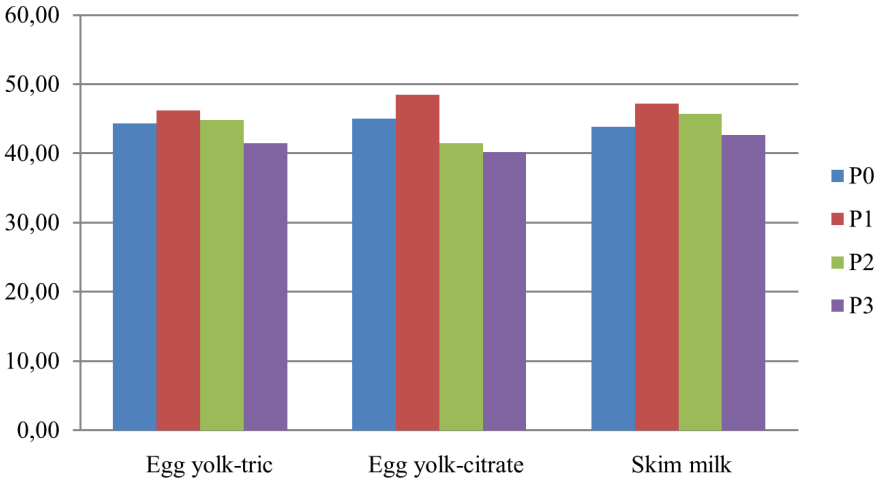

Figure 1. The average percentage of motility of Brahman cow spermatozoa after freezing

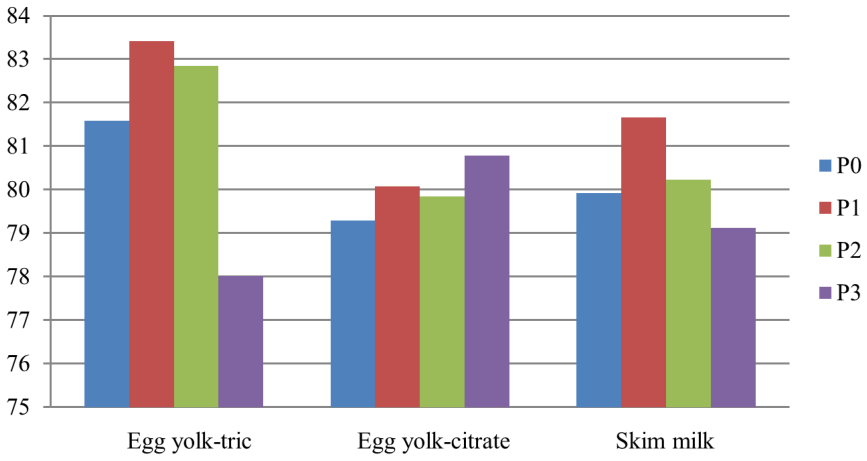

Figure 2. The average viability of Brahman cattle after freezing

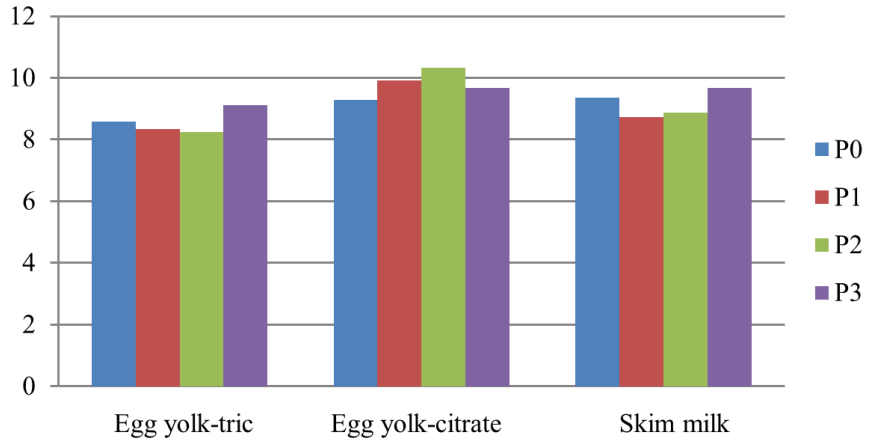

Figure 3. The average percentage of abnormalities of Brahman cow spermatozoa after freezing

The results showed that the P3 treatment had lower values in the TEY and SM extenders compared to P0, P1, and P2, likely due to an excessive dose of antioxidants, which may accelerate oxidative stress and even damage sperm cells during freezing. Oxidative reactions involving free radicals can damage cell membranes and DNA composition, potentially leading to cell death. Excessive antioxidant doses can influence the oxidation rate, causing the depletion of antioxidant activity, and in fact, excessive antioxidants can become pro-oxidants (Ames *et al*. 1993; Kunwar and Priyadarsini 2011). Spermatozoa are vulnerable to stress from ice crystal formation, osmotic pressure, and free radical formation during freezing and thawing. The results of this study are significantly higher than previous research with the addition of vitamin C and the same extender, which was around 44.65±2.71% (Abdillah 2018). Other studies have reported that the percentage of live spermatozoa post-thawing in the same extender with the addition of vitamin E was similarly high at 87.70±1.95% (Hartono 2008).

The analysis of variance showed that the addition of vitamin C, vitamin E, and the combination of vitamins C and E did not result in significant differences (P>0.05) in the TEY, CEY, and SM extenders. There were also no significant differences observed with the use of different extenders.

The lack of differences is attributed to the fact that all three extenders utilized egg yolk and glycerol, which help prevent cold shock. Nur *et al*. (2023) reported that the lipoproteins and lecithin contained in egg yolk can stabilize spermatozoa membranes, thereby preventing abnormalities caused by freezing. The frozen Brahman cattle semen demonstrated good quality for use in insemination, as the abnormality rate was below 10%. According to BSN (2017), the abnormality rate of spermatozoa should be less than 20% when using fresh or frozen semen for artificial insemination programs.

CONCLUSION

Based on the research results and data analysis, it can be concluded that the addition of vitamin C to the Tris Egg Yolk, Citrate Egg Yolk, and Skim Milk extenders showed the best results in terms of motility, percentage of live spermatozoa, and spermatozoa abnormalities in Brahman cattle after freezing.

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