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Effect of Guava Filtrate Supplementation in Tris and Citrate-Based Extenders on Spermatozoa Quality of Brangus Bull after Sex-rest

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Abstract | The objective of this study was to determines the effect of guava fruit filtrate (GF) supplementation in Tris and Citrate-based extenders on spermatozoa quality of Brangus bull after sex-break. Semen samples (twelve ejaculates) were diluted in Tris-egg yolk (TEY) without GF (control group, T1), TEY with 10% GF (T2), Citrate-egg yolk (CEY) with 10% GF (T3), Citrate with 10% egg yolk and 10% GF (T4), then chill-storage. The variables quality evaluated include spermatozoa progressive motility, visibility, and abnormality. Spermatozoa quality was observed every 24 hours until the spermatozoa motility reached at least 40%. The results showed that progressive motility and viability on the fourth day storage in extender Citrate-egg yolk (CEY) with 10% egg yolk and 10% GF were 50.4 ± 1.6% and 57.1 ± 1.6%, significantly higher (P<0.05) than control, TEY with 10% GF and Citrate with 10% egg yolk. The spermatozoa abnormality was 12.9 ± 0.3%, the lowest in extender Citrate with 10% egg yolk and 10% GF on the fourth-day storage. In conclusion, supplementation of GF to extender improves the spermatozoa quality of Brangus bull after sex activity break for a long time. Moreover, the best quality of spermatozoa is found in supplementing 10% GF to Citrate with 10% egg yolk extender.

Keywords | Brangus bull, Chilled storage, Extender, Guava, Semen

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INTRODUCTION

Brangus has grown in several regions for transfer genetics superior to local cow through artificial insemination (AI), including in West Nusa Tenggara Province. However, in recent years the using of Brangus bull semen has been limited or even stopped in several areas of West Nusa Tenggara Province. According to the Banyumulek Artificial Insemination Center, there are still a few bulls that need to be maintained and used for AI. As an initial step of implementation, it is necessary to evaluate and preserve the quality of the Brangus bull spermatozoa.

The spermatozoa quality must be maintained for a long time after being outside the body of livestock by an extender that is necessary and not harmful to the viability of spermatozoa. The extenders are generally based on Tris (hydroxymethyl)–aminomethane or Sodium-citrate added with egg yolk. Egg yolks contain lecithin and phospholipids, which protect spermatozoa from cold stress, and low-density lipoprotein (LDL) that maintains spermatozoa membrane phospholipids during semen processes. Phospholipid fractions cause the interaction between LDL in egg yolk and spermatozoa plasma membrane that decrease the binding of proteins in seminal plasma to spermatozoa and reduce the phospholipid efflux from spermatozoa membranes. However, several weakness of using egg yolk are the risk of bacterial contamination such as *Es-*

cherichia coli which negatively affect to fertilization capacity of the contaminated semen (Bustani and Baiee, 2021; Nguyen et al., 2019).

Extenders based on commercial synthetic and an animal materials tend to be at risk of contamination with bacteria or mycoplasma, and easily damage during storage and harm spermatozoa (Delgado et al., 2018). In addition, egg yolks contain substances that interfere with biochemical and metabolic tests, can inhibit respiration, and potentially reduce spermatozoa motility (Pillet et al., 2011; Sakr et al., 2021). Tris-egg yolk proteins could also be phosphorylated by the spermatozoa during the incubation and attach to the sperm membranes so that adhering to the sperm membrane and limiting their movements (Bertuzzi et al., 2020). Therefore, the use of egg yolk in the extender should be reduced, and supplemented by non-animal materials that contain antioxydants.

All biological membranes have a lecithin to limiting activation of superoxide dismutase (SOD) as membrane antioxidant enzyme, so prevent the membranes damage by reactive oxygen species (ROS) (Zhang et al., 2021). Guava fruit (*Psidium guajava* Linn) is a source of natural non-enzymatic antioxidants such as vitamin C or ascorbic acid (AA), lutein, lycopene and zeaxanthin **(**Rahmat et al., 2006**)**. The content of vitamin C in guava fruit ranges from 180 – 300 mg vitamin C per 100 g of fruit (Angulo-Lovez et al., 2021), 142.55 mg/100 g in the leaves (Kumar et al., 2021).

According to Mittal et al. (2010), the supplementation of 5 mM vitamin E or 5 mM vitamin C into a semen extender can significantly reduce the percentage of abnormal spermatozoa after storage. Using of vitamin C (ascorbic acid) at the correct dose can regulate the utilization of vitamin E (α-tocopherol) by cells (spermatozoa). Decreasing levels of ascorbic acid is a risk factor for normal morphology of spermatozoa and idiopathic infertility in males (Colagar and Marzony, 2009).

Ascorbic acid (AA) indirectly regulates and maintains the antioxidant α-tocopherol in an active state to reduce the sensitivity of spermatozoa to lipid peroxidation with the support of dehydroascorbate reductase. Deficiency of vitamin C or E can cause oxidative stress (Aitken and Roman, 2008). Vitamin C is a critical biological antioxidant in cells and plays a role in redox tagging responses, as a co-factor in enzymatic reactions and scavenge or capture free radicals. The vitamin C is transported into cells in an oxidized form, namely DHA through facilitative glucose transporters (Gluts), then reduced rapidly in cells and accumulated as ascorbic acid (AA) (Carcamo et al., 2004). Adding vitamin C in extender maintained motility of chilled semen at 5° C (Achi et al., 2018), and benefic effects on motility of cryopreserved semen (Pinto et al., 2020). Therefore, it is necessary to try supplementation GF in Tris and Citrate-based extender on spermatozoa quality of Brangus bull after sex-break for more than a year.

MATERIALS AND METHODS

The research material was the semen of three Brangus bulls aged 4.5 to 6.5 years belonging to the Artificial Insemination Center of West Nusa Tenggara Province at Banyumulek. The semen was collected with an artificial vagina each six time per bull. The ejaculate obtained was taken to the Reproduction Laboratory, Faculty of Animal Husbandry, University of Mataram, for evaluation macroscopically and microscopically. The distance of the laboratory from the AI center is about 7 km by freeway, which takes about 8 to 10 minutes. Experimental animals in this study have received approval from the Animal Ethics Committee with the Register Number: 09/UN18.F2/EC/2021.

The first stage of the study was to assess the quality of fresh semen based on the results of the macroscopic and microscopic evaluation. The leading indicators used for the feasibility standard of fresh semen for processing in this study were semen volume ranging from 2 to 7.5 ml, progressive motility and viability of spermatozoa at least 70%, maximum abnormality 20% and minimum concentration 500 million cells per ml of semen, in accordance with Ghirardosi et al. (2018), Andrabi et al. (2008), Asadopour et al. (2011) and Suhardi et al. (2020). The second stage used an experimental method with a completely randomized design (CRD) consisting of four extender treatments for chilled storage. Treatment T1 consisted of tris-egg yolk (TEY) without GF as a control group, T2 was TEY with 10% GF, T3 was Citrate-egg yolk (CEY) with 10% GF, and T4 was Citrate with 10% EY and 10% GF (a half of EY was substitute with GF). Each group of semen samples was repeated six times.

Preparation Of The Standard Stock Of Gf

Guava was cleaned with water at 37 °C dried with a tissue, juiced, and then dissolved with aquabidest with a ratio of 1: 2. The solution was centrifuged for 10 minutes at a speed of 3500 rpm. The supernatant resulting from centrifugation was filtered with a millipore membrane (Sartorius stedim Minisart®) at 0.42 µm and continued at 0.2 µm. The filtrate (filtration liquid) obtained pasteurized in hot water at 60 °C for 2 minutes (Menchaca et al., 2005; Mollineau et al., 2011). After pasteurization, the GF is stored in the refrigerator at 5 °C using a glass tube or in the freezer using a 1.5 mL volume of a mini tube (Sumadiasa et al., 2015).

Preparation Of Tris (Hydroxymethyl)-Aminomethane Buffer

A total of 4.028g of tris (hydroxymethyl)-aminomethane and 1.0g of fructose were put into a 150ml Erlenmeyer tube, then added by 100ml aqua destilata and heated at 90 ^{O}C for 10 minutes (Modified from Schafer-Somi et al., 2021). The buffer solution is kept at 32 ^{O}C temperature, added by 1000µg streptomycin and/or 1000 IU penicillin per ml of extender then homogenized with a magnetic stirrer. The buffer was added with citric acid drop by drop sequentially, so the pH reached between 6 – 7, then stored in refrigerator at 5 ^{O}C until used (Sukirman et al., 2019; Raheja et al., 2018).

Preparation Of Sodium Citrate Buffer

A total of 2.8g of Sodium citrate (Na-itrate) and 0.5g of fructose were put into Erlenmeyer and added by 100 ml of aquadestilata. The solution was heated while stirring until boiling (90 ^{O}C temperature) for 10 minutes (Modified from Nor-Ashikin and Abdullah, 2011). The solution was heated while stirring until boiling (temperature 90 ^{O}C) for 10 minutes. The solution was kept at 32 ^{O}C, and then added 1000 µg (0.1g) streptomycin and/or 1000 IU (0.06g) penicillin per ml of extender. The buffer solution was homogenized with a magnetic stirrer, then stored in a refrigerator at 5 ^{O}C until used.

Preparation Of Treatment Extender

A total of 4 sterile test tubes (10 ml in volume) prepared and labeled: namely T1, T2, T3 and T4, for treatments 1 to 4. The test tubes with label T1 were filled with 80% tris and 20% egg yolk (TEY) without GF. The T2 tube was filled with TEY with 10% GF, the T3 tube was filled with Citrate-egg yolk (CEY) with 10% GF, and the T4 tube was filled with Citrate with 10% EY and 10% GF. The extender solution in each test tube was homogenized with a magnetic stirrer and then centrifuged at 3500 rpm for 5 minutes. The solution supernatant was moved to new test tubes for each treatment and then stored at 5 ^{O}C in a refrigerator for chilled storage.

Semen Collection And Evaluation

Semen of three Brangus bulls was collected using an artificial vagina twice a week. The ejaculate was immediately taken to the Reproduction Laboratory of the Animal Husbandry Faculty, University of Mataram, for detailed macroscopic and microscopic assessment. The evaluated semen was immediately diluted in each prepared medium treatment (T1, T2, T3, and T4) according to the concentration of spermatozoa. Furthermore, the treatment semen was incubated at 5 ^{O}C in a refrigerator (chilled storage) with a water jacket for further evaluation.

Evaluation Of The Spermatozoa Quality During Chilled Storage

The sample of each semen was evaluated as soon as diluted and incubated at 5 ^{O}C in a refrigerator. Evaluation was repeated every 24 hours under 400x magnification of a phase contrast microscope until the spermatozoa motility reaches at least 40%. Evaluation was done at 32 ^{O}C on microscope hot-plate. The parameters evaluated included the variables of progressive motility, viability, and abnormality of spermatozoa. Progressive motility refers to spermatozoa that are swimming in a mostly straight line or large circles. Viability is defined as the percentage of live spermatozoa in a semen sample. Abnormality is spermatozoa have head or tail defects, such as a large or misshapen head or a crooked or double tail.

Data Analysis

The data was analyzed by Analysis of Variance (ANOVA) using Statistical Product and Service Solutions (SPSS) software ver.20 in Multivariate of General Linear Model with Duncan Equal Variances Assumed test.

RESULTS AND DISCUSSION

The Quality Of Fresh Semen

Generally, the minimum requirements for a probable fertile of bull semen should include over than 500 million spermatozoa per ml, more than 50% of motile spermatozoa make forward progression, and more than 80% of the spermatozoa conform to normal morphology (Suhardi et al., 2020). Prolonging sexual break is thought can affect semen quality, including volume, concentration, viability, abnormality, and progressive motility of spermatozoa. The reason is Breaking the sexual activity for a long time causes the testicles to become inactive, low positive feedback on the secretion of testosterone hormone that reduces bull's libido level, and affecting the production and quality of spermatozoa (Sumadiasa et al., 2017).

The semen used in this study had average quality criteria as the requirements for artificial insemination programs, except for progressive motility which less than 70%. Table 1 shows the average quality of the three bulls of fresh semen.

Table 1 shows that, the volume of semen obtained was lower than that reported by some previous researchers, namely 5.48 to 6.44 ml (Bhave et al., 2022), 5.20 ± 0.08 ml with variation from 5.12 ± 0.15 to 5.98 ± 0.09 among the ages 3 – 9 years (Sankhi et al., 2019), 4.84 ± 0.01 ml in Kankrej bulls semen (Patel and Siddiquee, 2013), and 10.5 ml (Hossain et al., 2012). This difference was presumably caused by the sexual break of Brangus bulls for several years, and poor quality of the woof. Breed had a highly significant effect to semen volume of bulls, wherein the av

Table 1: The quality of fresh semen in three groups of Brangus bull (n = 18).

Quality indicators	Mean semen quality in the group		
	I	II	III
Volume (mL)	4.89 ± 1.88	4.4	2.15
Color	Creamy white to milky white	Creamy white to milky white	Creamy white to milky white
Consistency	Medium to rather thick	Medium to rather thick	Medium to rather thick
Degree of acidity (pH)	6.23 ± 0.08	6.23 ± 0.07	6.28 ± 0.13
Mass motility	3^+/D	3^+/D	2^+ to 3^+/SD
Progressive motility (%)	68.33 ± 2.58	60.00 ± 0.00	56.00 ± 5.48
Concentration (10^6)	1448.25 ± 221.38	2143.14 ± 288.55	1467.33 ± 162.86
Viability (%)	80.83 ± 3.43	76.25 ± 7.83	66.60 ± 4.77
Abnormalities (%)	4.17 ± 0.75	7.88 ± 3.60	17.00 ± 4.30

Explanation: D = densum (solid); SD = semi densum (slightly dense)

erage semen volume of Bali bulls (5.628 ± 1.815 ml), Madura bulls (5.734 ± 1.740 ml) and Simmental bulls (6.635 ± 2.341 ml) (Novianti et al., 2020).

The semen color obtained was creamy white to milky white, the typical color of normal fresh semen. The level of thickness or consistency of the semen ranges from slightly thick to thick, and the pH was normal about 6.2. Generally, the pH of a bull's semen was at around 7.0 with variations depending on the individual bull. According to Santoso et al. (2021), the pH ranges from 6.3 to 6.46 in Pasundan bull. Increasing the pH of bull semen decreased viability, longevity, and motility of spermatozoa. Bacterial methabolism changes the pH conditions, characteristics of seminal plasma and affect the function of spermatozoa. These reported was close to the findings of Patel and Siddiquee (2013).

Semen quality is the main factor that determining fertility and reproductive efficiency of bull (Kurmi et al., 2018). The mass motility of spermatozoa in this study was in the average of 3^+ (+++), which indicates the number of spermatozoa that were moving together very dense (densum), estimated more than 90% spermatozoa moving forward (progressive). This result followed by Arefin et al. (2022), 62.11 ± 0,56% in Red Chittagong Cattle semen and Diansyah et al. (2022), 94.94 ± 2.75%.

Progressive motility of spermatozoa is one of important factors to support the fertilization. In this study, the progressive motility of spermatozoa ranges from 60% to 70%, in accordance with reported by Widiarta et al. (2019), 69%, Pause et al. (2022), maximum 80%, and lower than reported by Nurcholis et al. (2021), 81,1 ± 1.42%. The percentage of live spermatozoa were from 60% to 85%, slightly lower than reported by Diansyah et al. (2022), 95.73 ± 2.15. This is due to the sex-break of the bull for a long period, more than two year, and was directly proportional to pH of semen as shown in Table 1.

The normal spermatozoa more than 80% was found in semen group III, more than 90% in group II and more than 95% in group I. This results was in accordance with reported by Arefin et al. (2022), 87.27 ± 0.31% in Red Chittagong Cattle (RCC), Tarig et al. (2021), 98.42 ± 0.31 in Tris-egg yolk, and higher than Jae-Wook et al. (2022), 58.5 ± 4.1% to 71.1 ± 7.2% in Jeju Black Cattle. Difference of normal spermatozoa caused by breed and individual livestock such as in beef and dairy bulls was 76 ± 8% and 87 ± 6% (Morrell et al., 2018).

The concentration of spermatozoa obtained ranging of 1152 to 2430 x 10^6 per ml of semen. This concentration was higher than the standard feasibility process (1000 x 10^6/ml) in this study and reported by Abavisani et al. (2013), Hossain et al. (2012), 0.81 x 10^9/ml, Sarder (2007), 1.32 x 10^9/ml, and Patel and Siddiquee (2013) was 1.25 x 10^9.

Quality Of Semen After Preservation

Semen preservation was performed in liquid form (liquid semen) by storing the semen at 5 ^{O}C in a refrigerator. The motility of preserved spermatozoa in Citrate-based extender containing 10% EY and 10% GF was higher than 40%, and more than motility in T1, T3 and T2 from day O to day five of storage. Table 2 presents the progressive motility of preserved spermatozoa during storage in Tris or Sodium Citrate-egg yolk supplemented with GF.

Based on Table 2, the applied treatment showed that spermatozoa motility in T4 (Citrate with 10% EY and 10% GF) was very significantly higher than treatment T2 ($P < 0.01$), and significantly different with treatment T3 ($P < 0.05$), but did not significantly different with control (T1) ($P > 0.05$) until day sixth of storage. As well as on day seven, the combination extender of 80% Sodium Citrate added by 10% EY and 10% GF was more suitable for preserving Brangus bull spermatozoa compared to T2, T3 and T1.

Table 2: The average of Progressive Motility of Brangus bull Spermatozoa During Preserved in Tris or Sodium Citrate Egg Yolk Supplemented with GF (n = 18)

Observation Day	Treatments				SEM	*p*-value
	T1	T2	T3	T4		
1	63.3 ± 1.0[a]	58.1 ± 1.2[b]	60.7 ± 1.2[ab]	65.3 ± 0.9[ac]	1.071	0.001
2	60.3 ± 1.2[a]	56.1 ± 1.4[b]	56.2 ± 1.3[b]	58.4 ± 0.7[a]	1.267	0.005
3	52.9 ± 1.4[a]	48.3 ± 1.6[b]	48.8 ± 1.4[b]	54.5 ± 1.3[a]	1.267	0.005
4	49.5 ± 1.7[a]	44.3 ± 1.6[b]	46.7 ± 1.4[ab]	50.4 ± 1.6[ac]	1.582	0.003
5	47.1 ± 3.8[a]	42.1 ± 3.1[b]	43.6 ± 1.8[ab]	49.3 ± 2.0[c]	1.376	0.001
6	46.1 ± 1.3[a]	38.9 ± 1.6[a]	42.5 ± 0.9[a]	48.3 ± 0.9[a]	2.774	0.271
7	40.0 ± 3.1[ab]	32.9 ± 2.9[a]	34.3 ± 3.5[a]	43.6 ± 1.4[b]	2.835	0.045

[ab]Different superscripts within each row indicate significant differences (P <0.05)
T1 = Tris-egg yolk (TEY) without GF as a control group, T2 = TEY with 10% GF, T3 = Citrate-egg yolk (CEY) with 10% GF, T4 = Citrate with 10% EY and 10% GF

According to some researchers, actively moving spermatozoa have progressive motility in 50% to 80%. The range of motility is influenced by several factors such as age and breed, maturity of spermatozoa and quality of fresh semen (Bhakat et al., 2014). The highest percentage of progressive motility of spermatozoa (more than 40%) was found in Citrate with 10% EY and 10% GF (T4) on the seventh day of storage. Reducing a half proportion of egg yolk and replaced with GF makes the extender in T4 more preservative than other treatments. Egg yolks contain substances that interfere with biochemical and metabolic tests, can inhibit respiration, and potentially reduce spermatozoa motility (Pillet et al., 2011; Sakr et al., 2021).

Egg yolk proteins could also be phosphorylated by the spermatozoa during the incubation and attach to the sperm membranes so that adhering to the sperm membrane and limiting their movements (Bertuzzi et al., 2020). Therefore, the use of egg yolk in the extender should be reduced, and supplemented by non-animal materials that contain antioxydants, namely GF. Free radicals or reactive oxygen species (ROS) are compounds that can cause damage to spermatozoa during storage (Lobo et al., 2010). The damaged spermatozoa membrane cannot provide enzymes in the metabolic process, so the availability of energy to maintain motility, viability, and the integrity of the acrosomal hood is low. The natural antioxidants contained in GF are proven to protect spermatozoa from damage caused by free radicals.

The highest viability percentage of the spermatozoa on the seventh day of storage was found in Citrate with a 10% egg yolk extender supplemented by 10% GF (T4). This result was significantly (P<0.05) different with treatment T1, T3 and T2 (Tris-egg yolk without GF, Tris-egg yolk with 10% GF and Citrate-egg yolk with 10% GF). The combination of both substances mentioned T4 can be estimated to make a preservation and protection system for spermatozoa so that the viability, motility, and normality can be appropriately maintained. Table 3 present the mean percentage spermatozoa viability of Brangus bull preserved in Tris-egg yolk or Citrate-egg yolk supplemented by GF.

These study results indicate that GF was suitable to be added to the Citrate-egg yolk extender to preserve Brangus bull spermatozoa, especially in T4. Egg yolk acts as a macro-cryoprotectant, fungtions as a source of nutrition, and protects spermatozoa from cold stress (cold shock). Citrate is a buffering agent to neutralize pH in the extender. The enzymes and vitamins in the guava fruit (*Psidium guajava* Linn) can act as an antioxidant system that protects spermatozoa from reactive oxygen species (ROS) and prevents spermatozoa's structural and ultrastructural integrity (Sumadiasa et al., 2015).

In previous study, that substitution of 10% egg yolks with GF in Tris-egg yolk extender preserves the quality of pre-freezing and post-thawing of Bali bulls spermatozoa (Sumadiasa et al., 2018). Several antioxidants in GF were suitable to mix with lecithin, lipoproteins, and egg yolks phospholipids. Antioxidants and various essential components found in guava fruit are carotene, retinol equivalent, vitamins B1 and B2, niacin, fiber, vitamin C or ascorbic acid (AA), lutein, lycopene and zeaxanthin (Rahmat et al., 2006). Vitamin C in guava fruit ranges from 50 - 300 mg/100g of fresh weight (Thaipong et al., 2005) so can maintain the motility and viability of spermatozoa during storage. Previous studies on use of guava (GF) for spermatozoa preservation have been carried out with quite good results (Sumadiasa et al., 2015; Sumadiasa et al., 2017), however there have been no reports from other researchers.

Abnormality of spermatozoa in various breeds of a bull is different, i.e. 20.45 ± 16.60% in Angus, 20.64 ± 16.44% in Simental, 18.11 ± 12.61% in Charolais; 16.97 ± 14.56 in

Table 3: Viability of Brangus bull Spermatozoa During Preserved in Tris-egg yolk or Citrate-yolk supplemented by GF (n = 18)

Observation Day	Treatments				SEM	*p*-value
	T1	T2	T3	T4		
1	68.3 ± 1.1^{a}	64.1 ± 0.7^{b}	66.2 ± 0.8^{ab}	70.9 ± 1.0^{c}	0.889	0.001
2	65.7 ± 0.8^{a}	62.5 ± 0.8^{b}	63.9 ± 1.0^{ab}	67.8 ± 0.8^{ac}	0.823	0.001
3	58.7 ± 1.2^{a}	55.2 ± 1.2^{a}	56.3 ± 1.3^{a}	60.4 ± 1.2^{ac}	1.217	0.014
4	54.1 ± 1.6^{a}	49.5 ± 1.4^{b}	50.1 ± 1.4^{ab}	57.1 ± 1.6^{ac}	1.492	0.003
5	52.8 ± 1.5^{a}	49.3 ± 1.9^{ab}	50.4 ± 2.1^{a}	55.1 ± 1.6^{ac}	1.755	0.100
6	51.1 ± 3.7^{a}	47.1 ± 3.1^{a}	47.9 ± 1.9^{a}	53.1 ± 2.4^{a}	2.817	0.438
7	44.8 ± 3.1^{a}	38.9 ± 3.3^{ab}	39.8 ± 2.8^{a}	48.0 ± 1.5^{ac}	2.732	0.089

^{ab}Different superscripts within each row indicate significant differences (P <0.05)

Table 4: The averages Abnormality of Brangus bull Spermatozoa During Preserved in Tris-egg yolk or Citrate-yolk supplemented by GF (n = 18)

Observation Day	Treatments				SEM	*p*-value
	T1	T2	T3	T4		
1	12.1 ± 0.4^{a}	13.3 ± 0.4^{b}	11.3 ± 0.3^{c}	9.8 ±0.3^{d}	0.345	0.001
2	13.5 ± 0.4^{a}	14.9 ± 0.4^{b}	12.4 ± 0.3^{a}	10.7 ± 0.3^{c}	0.348	0.001
3	14.2 ± 0.3^{a}	15.4 ± 0.4^{b}	13.6 ± 0.3^{a}	11.8 ± 0.4^{c}	0.321	0.001
4	14.8 ± 0.3^{a}	15.9 ± 0.3^{b}	14.4 ± 0.3^{a}	12.9 ± 0.3^{c}	0.281	0.001
5	14.7 ± 0.3^{a}	15.7 ± 0.3^{b}	14.6 ± 0.3^{a}	13.4 ± 0.3^{c}	0.304	0.001
6	14.6 ± 0.4^{a}	15.6 ± 0.3^{b}	14.7 ± 0.3^{a}	13.2 ± 0.3^{c}	0.318	0.001
7	15.3 ± 0.3^{a}	16.0 ± 0.3^{a}	15.3 ± 0.1^{a}	14.0 ± 0.3^{b}	0.265	0.001

^{ab}Different superscripts within each row indicate significant differences (P <0.05)

Limousin (Menon et al., 2011). The abnormality of spermatozoa in this study followed those described by Ghiradosi et al. (2018), namely 7.19 ± 4.91% in dairy cattle and 15.83 ± 9.28% in beef cattle. The lowest abnormality of spermatozoa was found in T4 then followed with T2, T3, and T1. The active ingredients of GF in Citrate-egg yolk extender can suppress spermatozoa abnormality far below than maximum standard (20%). Table 4 shows the abnormality of Brangus bull spermatozoa during preserved in Tris-egg yolk or Citrate-yolk supplemented by GF.

The abnormality of spermatozoa may occur in the head, and a small part occurs in the middle (midpiece) and tail. The head of the spermatozoa is essential key to fertilization, carrying out the genetic material into the egg for cleavage and forming the identity of the offspring. During storage, spermatozoa's abnormality is preceded by ROS interference, are highly reactive chemicals formed from diatomic oxygen (O_2). Reactive oxygen species block the fertilization process by adhering to the egg cell (oocyte) and damaging the DNA genetic material, resulting in DNA fragmentation in the spermatozoa's head. However, addition of exogenous antioxidants to semen can protect spermatozoa against the harmful affected by ROS during the cryopreservation process (Fadl et al., 2022).

According to Trout (2012), LDL and phospholipids (lecithin) from egg yolk are protective components that can prevent cold shock by sticking to the spermatozoa membrane to form an interfacial layer. The result is increases phospholipid and cholesterol bonds in the plasma membrane of spermatozoa which form a complex with seminal plasma proteins, thus making the lipids in the cell membrane not function. The low density lipoprotein (LDL) can reduce the loss of phospholipids, grab toxins from plasma seminal proteins and stabilize the membrane (Manjunath et al., 2012; Akhter et al., 2010). However, egg yolks contain substances that interfere with biochemical and metabolic tests, can inhibit respiration, and potentially reduce spermatozoa motility (Pillet et al. 2011; Sakr et al., 2021).

Egg yolk proteins could also be phosphorylated by the spermatozoa during the incubation, attach and adhering to the membranes of spermatozoa so that limiting their movements (Bertuzzi et al., 2020). Therefore, the use of egg yolk in the extender should be reduced, and supplemented by non-animal materials that contain antioxydants, namely GF. Reducing a half proportion of egg yolk in the extender and replaced with GF makes the diluent in T4 more preservative than other treatments.

Overall, the best quality of spermatozoa in this study was found in Citrate with 10% egg yolk and 10% GF. It is because all the nutrients, energy, pH buffer, and antioxidants needed for preserved and protected the spermatozoa in those extender were fulfilled. Egg yolks contain sources of nutrients, energy, and LDL, which function as protective elements for the cell membranes of spermatozoa. Sodium Citrate is a buffer for the pH of the solution, and GF as a source of antioxidants to prevent damage to spermatozoa due to free radicals or ROS (Sumadiasa et al., 2018).

Guava fruit provides various natural antioxidants essential for protecting spermatozoa during preservation such as vitamins C, E and -carotene. Vitamin C can help neutralize chemicals or toxins in semen, transform cholesterol, reduce the rate of cholesterol catabolic reactions, and reduces the stickiness of the spermatozoa to cluster together so the spermatozoa can move and swim quickly in the extender. Supplementation of vitamin C to an extender showed benefic effects on spermatozoa motility (Pinto et al., 2020). Supplementation of antioxidants both enzymatic (superoxide dismutase and catalase), and non-enzymatic in extenders before refrigeration could reduce detrimental effect to spermatozoa (Silvestre et al., 2021). Non-enzymatic molecules such as glutathione, thioredoxin, vitamins D, E, and C is a mechanism to maintain motility and viability, and suppress spermatozoa abnormalities by preventing lipid peroxidation in semen (Trout et al., 2012; Zeitoun and Al-Damegh, 2015).

CONCLUSIONS

Based on the result of this research, sex break has no effect on the semen characteristics of Brangus bull, except at relatively low of fresh semen volumes. Quality of Brangus bull spermatozoa while chilled-storage in Tris and Sodium Citrate-based extender supplemented with GF is higher than without GF. The combination of 80% Sodium Citrate with 10% EY and 10% GF is the best formulation of four treatment extenders.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interests.

NOVELTY STATEMENT

Utilization of guava fruit filtrate in semen extender to improving spermatozoa quality of the bulls after sex activity break for long time. It is found that addition of 10% guava fruit filtrate to Citrate with 10% egg yolk extender can maintain the quality of bull spermatozoa until 7 days of storage and fit to applied for artificial insemination (AI).

AUTHORS CONTRIBUTION

All authors have developed the theory and supervised the research. I Wayan Lanus Sumadiasa, Enny Yuliani, and Lukman HY contributed to the sample collection, analysis calculations, and writing the final version of the manuscript.

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