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The Effect of the Combination of Glucose Concentration with the Type of Extenders on the Quality of Native Rooster Spermatozoa during Storage

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Abstract

This study aimed to find the best combination of glucose concentration with the type of extender to maintain progressive motility and viability of Native Rooster spermatozoa, which stored for 48 hours at 5°C. This study used a completely randomized design (CRD) factorial pattern of 4 x 3. The first treatment was a different type of extender: LE (lactated ringer egg yolk), LS (lactated ringer skim milk), CE (coconut water egg yolk) and CS (coconut water skim milk), while second treatment was glucose concentration (20mM, 50mM, and 80mM). Native Rooster semen diluted with a ratio of 1:10, then sperm was stored in the refrigerator (5°C). Progressive motility and viability were observed at 0, 24, 48, and 72 hours. The results showed the type of extender treatment had a very significant effect (P <0.01) on the viability of spermatozoa, which were stored 24-hour. The treatment of extender type, glucose concentration, and interaction of extender type with glucose concentration had a very significant effect (P <0.01) on the quality of spermatozoa, which were stored 48 hours and 72 hours. The highest progressive motility of spermatozoa was maintained up to 46.67 ± 2.89% and spermatozoa viability up to 74.68 ± 4.51% in WE 80mM glucose extenders, so the conclusion of this study is the combination of egg yolk coconut water with 80mM glucose is the best extender to maintain progressive motility and viability of spermatozoa of Native Rooster which is stored for 72 hours at 5°C.

Keywords: glucose, extenders, spermatozoa, storage, Native Rooster

A. Introduction

Native Rooster is one of the chicken types that were popular in Indonesia. However, its rearing system is still traditional in the way it is spread so that the genetic quality of Native

chickens is not controlled, so to overcome this, it is necessary to improve the genetic quality of Native chicken. Some attempts have been made by researchers to improve the genetic quality of chicken, one of them by utilizing the latest technology that has been done in other countries such as artificial insemination (AI). One of the crucial steps to be done before AI is semen dilution. When semen is not be diluted, the number of the hen that would be recipients for AI is less and cannot long last for storage. According to Blesbois (2012), the critical factors that support the success of in vitro storage, including in vitro media, must be compatible with spermatozoa cell life.

Based on the previous study, the extender commonly used in mammalian semen contains various substances, for example, egg yolk, skim milk, or coconut water, while the lactated ringer is used for poultry semen. Each type of extender has a different effect on the quality of spermatozoa. The study of Daramola's, Adekunle, Oke, Onagbesan, Oyewusi & Oyewusi (2016) stated that the protective effects of coconut water on the viability of cryopreserved spermatozoa of bucks. Duck and quail egg yolks can be an abundant source of DHA for boar semen cryopreservation (Kaeoket & Chanapiwat, 2013). Duck egg yolk can be used to Boer goat semen extender, and it is a 10% minimum concentration in diluted semen (Ihsan, 2011).

Study by Galarza, de Guevara, Beltrán-Breña, Sánchez-Calabuig, Rizos, López-Sebastián & Santiago-Moreno (2019) describe that skim-milk had a decisive role in the regulation of boar sperm motility by influencing sperm protein modifications changes as well as increasing the GAPDH activity, mitochondrial membrane potential, and intracellular ATP content (Fu, Li, Wang, Zhen, Yang, Li, & Li, 2017). Ringer's solution consists of various mineral salts that have buffering and isotonic properties, which can support the spermatozoa motility for a longer time (Danang, Isnaini & Trisunuwati, 2012). The use of lactated ringers as extender results in higher fertility and fertile periods in free-range chickens when compared with NaCl 0.9% extender (Ridwan & Rusdin, 2008).

The energetic metabolism is one of the primary sources of problems during and after in vitro storage at birds' sperm. At the time of ejaculation, avian sperm contains very few intra-cellular energy reserves, and energy substrates may be added to the extender to prolong sperm motility and viability in vitro (Blesbois, 2012). Glucose is a type of carbohydrate that is commonly added in mammalian semen extenders as an energy source. According to Qiu, Li, Xie, Li, Dong, Sun, Gao & Tan (2016), spermatozoa metabolize glucose during long-term liquid storage of semen, and this is important not only in the goat but also in other species. Previous researchers have proven the effectiveness of the combination of extenders with carbohydrates on the quality of poultry spermatozoa, for example, Rochmi & Sofyan (2019) found a diluent containing a mixture of coconut water, egg yolk, and fructose can be added to rooster sperm to increase spermatozoa motility and viability for up to 7 days when the cement samples are stored at 5 ° C.

The use of lactated ringer-glucose extender induces higher post-thawing turkey spermatozoa motility when compared to lactated ringer only (Kuzlu & Taskin 2017). According to the study of Mayesta, Trilaksana & Bebas (2014), motility of spermatozoa with 0.6% glucose treatment gave the best results to maintain the motility of Native Rooster spermatozoa on phosphate-egg yolk extenders stored at 3-5 ^{o}C. Based on this description the research conducted aims to find the best combination of glucose concentration with the type of extender (lactate ringer egg yolk, lactate ringer skim milk, coconut water egg yolk, and coconut water skim milk) to maintaining progressive motility and viability of Native rooster spermatozoa stored for 48 hours at 5 ^{o}C.

B. Methodology

1. Research Design

This study used a completely randomized design (CRD) factorial pattern of 4 x 3 with three replications (semen collection). The first factor is the type of extender, which consists of CE, CS, LE, and LS. The second factor is glucose concentration consisting of 20 mM, 50 mM, and 80 mM.

2. Research Procedures

a. Extenders preparation

The composition of extenders was lactated ringer (PT Widatra Bakti) mature coconut water, duck egg yolk, skim milk (Tropicana Slim), D(+)-glucose anhydrous (Merck), penicillin, streptomycin (PT Meiji Indonesian) and tris hydroxyl aminomethane (Merck). LE (1.5 ml egg yolk mixed with 8.5 lactated ringer, centrifuged 2000 rpm for 20 minutes), LS (9 ml lactated

ringer mixed with 1 gram skim milk), CE (1.5 ml egg yolk mixed with 8.5 coconut water, centrifuged 2000 rpm for 20 minutes), CS (9 ml of coconut water mixed with 1 gram of skim milk). Glucose was added according to the treatment namely 20 mM (0.036 gr/10 ml), 50 mM (0.09 gr/10 ml) and 80 mM (0.144 gr/10 ml). Each extender was added penicillin 1000 IU/ml and streptomycin 0.1 mg/ml. The pH of the extender was adjusted using the tris hydroxyl aminomethane until a pH of 7 was obtained.

b. Semen collection and treatment

Semen was collected from Native roosters aged more than one year using the massage method. Semen was diluted according to the treatments by comparison of sperm with extender 1:10. Then the liquid semen was stored in a refrigerator with a temperature of 5 ^{o}C.

3. Research Parameters

Progressive motility of spermatozoa (%) and viability of spermatozoa (%) were observed in this study using a 40x magnification light microscope at 0, 24, 48, and 72 hours.

4. The technique of Data Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) if found the effect of the treatment continued with the Duncan test.

C. Results Findings and Discussion

1. Sperm motility

Table 1. Progressive motility of Native rooster spermatozoa (%) with various extenders during liquid storage at 5 ^{o}C (Mean±SD)

Observation time (h)	Extender	Glucose concentration			Average
		20 mM	50 mM	80 mM	
0	LE	85.00±0.00	85.00±0.00	85.00±0.00	85.00
	LS	83.33±2.89	86.67±2.89	81.67±2.89	83.89
	CE	86.67±2.89	86.67±2.89	86.67±2.89	86.67
	CS	86.67±2.89	86.67±2.89	86.67±2.89	86.67
	Average	85.00	86.25	85.00	
24	LE	78.33±2.89	76.67±2.89	75.00±0.00	76.67
	LS	76.67±5.77	81.67±2.89	76.67±2.89	78.33
	CE	80.00±0.00	80.00±5.00	78.33±2.89	79.44
	CS	80.00±0.00	78.33±2.89	73.33±5.77	77.22
	Average	78.75	79.17	75.83	
48	LE	8.33±2.89^{aW}	6.67±2.89^{aW}	5.00±0.00^{aW}	6.67
	LS	8.33±2.89^{aW}	16.67±2.89^{bX}	65.00±5.00^{cX}	30.00
	CE	75.00±0.00^{aX}	71.67±5.77^{aY}	71.67±2.89^{aX}	72.78
	CS	8.33±2.89^{aW}	8.33±2.89^{aW}	68.33±5.77^{bX}	28.33
	Average	25.00	25.83	52.50	
72	LE	0.00±0.00^{aW}	0.00±0.00^{aW}	0.00±0.00^{aW}	0.00
	LS	0.00±0.00^{aW}	0.00±0.00^{aW}	11.67±2.89^{bY}	3.89
	CE	26.67±2.89^{aX}	35.00±5.00^{bX}	46.67±2.89^{cZ}	36.11
	CS	0.00±0.00^{aW}	0.00±0.00^{aW}	6.67±2.89^{bX}	2.22
	Average	6.67	8.75	16.25	

Description: Different lowercase letters (a, b, c) on the same line show very significant differences (P<0.01). Different capital letters (W, X, Y, Z) in the same column show very significant differences (P<0.01). SD = Standard deviation, LE = lactated ringer + egg yolk, LS = lactated ringer + skim milk, CE = coconut water + egg yolk dan CS = coconut water + skim milk.

a. Effect of type of extender

The use of the various kinds of extenders was not influenced (P<0.01) the progressive motility of rooster spermatozoa, which are stored for 0 hours as well as 24-hour storage. The average motility that was observed at 0-hour storage was 83.89-86.25%, almost the same as previously obtained in Sentul crossbreed rooster spermatozoa, which was 81.67% using lactated ringer egg yolk glucose extender at 0-hour storage (Khaeruddin, Arifiantini, Sumantri & Darwati, 2016). The motility average at 24-hour storage was 76.67-79.44% also higher than the previous report of 67.08% (Khaeruddin, Arifiantini, Sumantri & Darwati, 2016), 38 % using ringer's extender (Danang, Isnaini & Trisunuwati, 2012) and between 50 to 60 % using lactated ringer with egg albumin (Nugroho & Saleh, 2016).

Semen storage for 48 hours to 72 hours caused differences (P<0.01) in motility between types of the extender. CE was generally able to maintain progressive motility of spermatozoa better than LE, LS, and ES. It might be because coconut water contains sugar and antioxidants that are not presented at a lactated ringer. According to Reddy & Lakhsmi (2014), coconut water contains 95% water, 5% sugar consisting of glucose, fructose, and sucrose. Coconut water contains antioxidants such as phenol and ascorbic acid (vitamin C) (Santos, Vanderson, Bispo, Adriano, Filho, Isabella, Pinto, Lucas, Danta, Daiane, Vasconcelos, Fabíula, Abreu, Danilo, Isaac, Florencio, Osmar, Marisa, Marisa, Medeiros, & Humberto, 2013). Sugar in coconut water can be a source of energy for spermatozoa, and antioxidants can prevent damage to spermatozoa caused by free radicals. The results of Dwitarizki, Ismaya & Asmarawati's (2015) study stated that the addition of duck egg yolk in coconut water extenders increased the motility of Garut ram spermatozoa. LDL in the egg yolk interacts with BSP proteins in the seminal plasma factors and is responsible for sperm protection (Manjunath, 2012). Besides, egg yolks can be an additional source of energy for spermatozoa. It is consistent with the opinion of Ponglowhapan, Essen-Gustavsson & Forsbeg (2004), which states that the addition of egg yolk to extenders is considered necessary, even though this increases the glucose content because egg-yolk is an essential ingredient in semen extenders. And in 20% egg-yolk solution in distilled water, the glucose concentration was found to be 3-4 mM (Ponglowhapan, Essen-Gustavsson & Forsbeg, 2004).

b. Effect of glucose concentration

Based on the results of the study (Table 1), differences in glucose concentrations did not induce differences in progressive motility at 0 hours and 24-hour storage. The average motility 85-86.25% in this study was close to the results obtained by Mayesta, Trilaksana & Bebas (2014), which was 89% with the addition of glucose into phosphate egg yolk extenders. Differences in motility began to appear at 48, and 72 hours of storage, increasing glucose concentrations tend to increase sperm motility

The result of this study is similar with the research of Ponglowhapan, Essen-Gustavsson & Forsbeg (2004) that 1-3 days storage did not produce a difference in motility of canine spermatozoa between the addition of glucose concentration of 10 mM to 70 mM, but at the storage time of 4-23 days resulted a significant difference, increase ten mM to 70 mM was followed increase sperm motility. The results of this study are also similar to reported by Mayesta, Trilaksana & Bebas (2014) that an increase in glucose concentration of 0.3 w/v% to 0.6 w / v% increased motility of Native rooster spermatozoa at a storage 5 ^{o}C.

Glucose can be a good source of energy for spermatozoa during storage. Spermatozoa utilize energy on motility, which is primarily in the form of intracellular ATP generated by oxidation of substrates, fructose, glucose, sorbitol, lactate, or pyruvate (Misro & Ramya, 2012). According to Qiu, Li, Xie, Li, Dong, Sun, Gao & Tan (2016), glucose and pyruvate are better than lactate in maintaining motility of goat spermatozoa. The energy metabolites available to sperm vary between species, but they generally consist mainly of sugars (primarily fructose or glucose) and fatty acids found in seminal plasma and the oviduct (Blesbois, 2012). Added by the opinion of Ponglowhapan, Essen-Gustavsson & Forsbeg (2004) that when the motility was depressed by cold storage, dog sperm utilized more glucose for other cellular activities than fructose.

c. Effect of interaction of the type of extender with glucose concentration

The interaction of the type of extender with glucose concentration had a very significant impact (P <0.01) on the motility of spermatozoa at 48 hours and 72 hours of storage. The use of 80 mM glucose in extender was able to maintain progressive motility better at 72 hours of storage than other treatments. The progressive motility of spermatozoa in CS extenders was higher dramatically than the progressive motility of spermatozoa in LS diluents at increasing glucose concentrations from 50 mM to 80 mM (Figure 1).

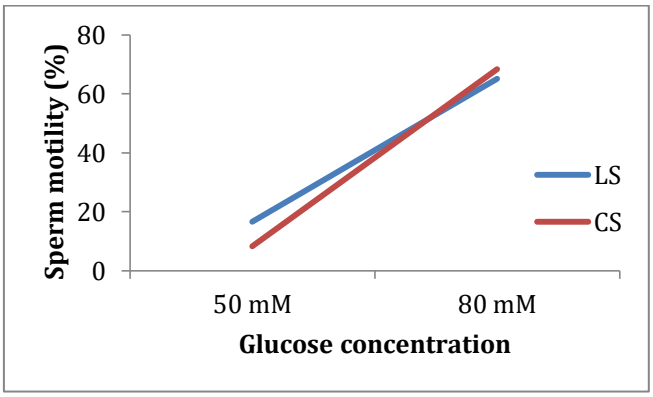

Figure 1. Graph of the interaction of glucose concentration with the type of extender on sperm motility in 48-hour storage at two levels of treatment.

CE extenders were able to maintain spermatozoa motility for up to 72 hours of storage, the combination of CE with 80 mM glucose induces the best spermatozoa motility. Whereas LE, LS 20 mM glucose, LS 50 mM glucose, CE 20 mM glucose, and CE 50 mM glucose extender were only able to maintain the progressive motility of spermatozoa for up to 48 hours. The spermatozoa motility in 80 mM glucose CS extender was 68.33 ± 5.77% at 48 hours storage, which was higher than previously obtained using coconut water + 9% skim milk extender which was 43.73% in the same type of chicken (Khaeruddin & Srimaharani, 2019).

2. Sperm viability

The average of spermatozoa viability at 5°C in the 0 hours storage was the range from 91.97 to 93.34%. In storage times of 24, 48, and 72 hours, sperm motility gradually decreased respectively in all extenders. At 72 hour storage, the average percentage of sperm viability was less than 75% in all extenders. Average viability values after dilution are presented in table 2.

Table 2. Progressive viability of Native rooster spermatozoa (%) with various extenders during liquid storage at 5 °C (Mean±SD)

Observation time (h)	Extender	Glucose concentration			Average
		20 mM	50 mM	80 mM	
0	LE	93.32±2.13	91.72±1.02	95.00±0.70	93.34
	LS	90.40±3.49	95.29±4.54	90.21±2.61	91.97
	CE	93.12±2.36	93.57±0.47	92.94±2.22	93.21
	CS	92.99±4.13	91.29±3.10	93.07±2.89	92.45
	Average	92.46	92.96	92.80	
24	LE	81.81±1.54	81.46±2.88	78.40±2.05	80.56^{A}
	LS	82.23±4.51	86.35±2.00	82.11±4.86	83.56^{BC}
	CE	84.22±0.89	86.66±2.92	84.92±1.37	85.26^{C}
	CS	82.45±0.57	81.95±1.89	81.98±2.03	82.13^{AB}
	Average	82.68	84.11	81.85	
48	LE	$38.93±1.53^{bY}$	$20.95±1.80^{aW}$	$19.52±0.92^{aW}$	26.47
	LS	$32.82±1.84^{aX}$	$47.47±3.06^{bX}$	$69.32±4.21^{cX}$	49.87
	CE	$80.76±0.56^{aZ}$	$83.70±1.97^{aY}$	$78.82±4.11^{aY}$	81.09
	CS	$12.77±1.25^{aW}$	$19.38±0.83^{bW}$	$71.70±3.57^{cX}$	34.61
	Average	41.32	42.87	59.84	
72	LE	$16.79±1.57^{cY}$	$10.65±0.58^{bX}$	$4.36±0.97^{aW}$	5.47
	LS	$7.09±0.70^{aX}$	$10.93±0.70^{bX}$	$30.36±2.30^{cY}$	16.13
	CE	$42.32±2.03^{aZ}$	$68.50±0.98^{bY}$	$74.68±4.51^{cZ}$	61.83
	CS	$3.41±0.83^{aW}$	$4.95±0.46^{aW}$	$12.69±1.53^{bX}$	7.02
	Average	17.40	23.76	30.52	

Description: Different lowercase letters (a, b, c) on the same line show very significant differences (P<0.01). Different capital letters (W, X, Y, Z) in the same column show very significant differences (P<0.01). SD = Standard deviation, LE = lactated ringer + egg yolk, LS = lactated ringer + skim milk, CE = coconut water + egg yolk dan CS = coconut water + skim milk.

a. Effect of type of extender

The kind of extender does not affect the viability of spermatozoa at 0-hour storage but changed at 24 hours, 48 hours, and 72-hour storage. CE extenders induced better viability than LE and CS (Table 2); this might be due to the CE content having a higher glucose content so that it allows spermatozoa to survive longer. Viability of spermatozoa in previous studies using skim coconut milk 9% in 24-hour storage was 79.90% (Khaeruddin & Srimaharani, 2019) was lower than in this study which was 82.13%, probably due to the presence of glucose content in this study. The viability average at 24-hour storage in the present study was also higher than the report of Danang, Isnaini & Trisunuwati (2012) that found 55.4% in ringer's extender.

b. Effect of glucose concentration

Glucose concentration in diluent affects the viability of spermatozoa in 48 hours and 72 hours of storage in some types of diluents. In general, 80 mM glucose concentration results in higher spermatozoa viability. Spermatozoa viability in 24 hours is in the average range of 81.85-84.11%, almost the same as that obtained by Mayesta, Trilaksana & Free (2014), which is 83.17-84.67% at the same time storage. Apart from being an energy source, sugar also has other functions for spermatozoa. Purdy (2006) stated that sugar is known to increase the osmotic potential of cells and protect the membrane from chilling-induced injury (Purdy, 2006).

c. Effect of interaction of the type of extender with glucose concentration

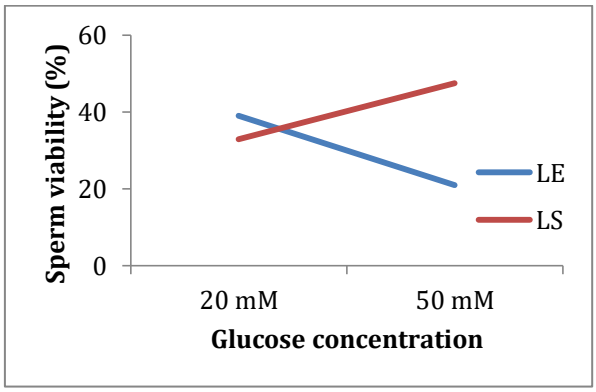

Figure 2. Graph of the interaction of glucose concentration with the kind of diluent on the viability of spermatozoa at 48 hours storage at each of the two treatment levels.

The interaction of the type of extender with glucose concentration had a very significant effect ($P < 0.01$) on the motility of spermatozoa at 48 hours and 72 hours of storage. The interaction graph (Figure 2) shows that the addition of 20 mM glucose-induced spermatozoa viability in RK extenders slightly higher than spermatozoa viability in LS extenders, but at 50 mM glucose concentrations induced much higher viability in LS when compared to LE at 48 hours of storage. It shows that the LS with 50 mM glucose extenders combination is better than a combination of the 50 mM glucose into LE extenders.

LE, LS, and CS extenders were still able to maintain the viability of spermatozoa within 72 hours of storage, but the spermatozoa have almost no progressive movement of spermatozoa but vibrate. CE is an excellent extender in storage for up to 72 hours, when combined with 80 mM glucose, can produce the best viability of spermatozoa. ROS accumulation can occur during semen storage when there are no antioxidants in the semen extender. According to Santos *et al.* (2013), coconut was able to reduce the concentration of intracellular ROS when compared to ascorbic acid. Coconut water contains L-arginine, ascorbic acid, and some minerals, which include calcium, magnesium, and potassium that may be vital for the sperm (Sandhya & Rajamohan, 2014).

D. Conclusion

The combination of egg yolk coconut water with 50 mM glucose is the best extender in maintaining progressive motility and viability of Native rooster spermatozoa, which are stored for 72 hours at 5^{o}C.

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