Effect of Carbohydrate Type and Phenotype on the Quality of Post-Thawing Frozen Semen of KUB Chicken

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ABSTRAK

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Ayam Kampung Unggul Balitbangtan (KUB) merupakan ayam yang memiliki fenotip yang berbeda-beda. Dilaporkan bahwa fenotip ayam berhubungan dengan kualitas semen. Penelitian ini bertujuan untuk mengetahui karakteristik dan kualitas *post-thawing* semen ayam KUB dengan fenotip berbeda yang dibekukan dalam pengencer ringer laktat kuning telur (RLEY) dengan penambahan fruktosa atau glukosa. Semen dikoleksi dengan metode massase dari 20 ekor ayam KUB dengan fenotip jengger tunggal dan warna bulu hitam atau coklat tua dengan warna leher bulu merah (SCNR), jengger tunggal warna bulu hijau-hitam dengan warna leher bulu putih (SCNW), jengger *pea* dan hitam atau gelap warna bulu coklat dengan warna leher bulu merah (PCNR), dan jengger *pea* warna bulu hijau-hitam dengan warna leher bulu putih (PCNW). Semen dari setiap fenotip ayam dibagi menjadi tiga bagian, atau dibekukan dalam tiga jenis pengencer yaitu RLEY, RLEY+fruktosa (RLEYF), dan RLEY+glukosa (RLEYG). Motilitas sperma paling tinggi ditemukan paling tinggi pada pengencer dengan penambahan glukosa pada fenotip SCNR dan PCNW ($P<0.05$). Viabilitas sperma tertinggi ditunjukkan pada pengencer RLEYG pada fenotip PCNW ($P<0.05$). Abnormalitas tertinggi ditemukan pada pemgencer RLEY dan RLEYF pada kelompok SCNW, PCNR, dan PCNW, sedangkan pada kelompok RLEYG hanya ditemukan pada kelompok PCNR. Recovery rate (RR) tertinggi ditunjukkan oleh kelompok RLEYG pada fenotip SCNR. Dari hasil penelitian ini dpaat disimpulkan bahwa kualitas semen pasca thawing dipengaruhi oleh jenis glukosa dan fenotip ayam dan yang terbaik ditemukan pada pengencer dengan penamban glukosa dalam fenotip SCNR.

Kata Kunci: Semen Ayam, Jengger, Pengencer, Suplementasi Media, Pembekuan

ABSTRACT

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The superior Balitbangtan Kampung Chicken (KUB) chickens have different phenotypes. It was reported that the chicken phenotype was related to semen quality. This study aimed to determine the post-thawing characteristics and quality of KUB chicken semen with different phenotypes frozen in Ringer's lactate egg yolk (RLEY) diluent with the addition of fructose or glucose. Semen was collected using the massaging method from 20 KUB chickens with a single comb phenotype and black or dark brown feather color with a red feather neck (SCNR), green-black single comb with white feather neck collar (SCNW), pea comb and black feathers or dark brown fur with a red neck (PCNR), and a green-black pea comb with a white neck (PCNW). Semen from each chicken phenotype was divided into three parts or frozen in three types of diluents: RLEY, RLEY+fructose (RLEYF), and RLEY+glucose (RLEYG). The highest sperm motility was found in the diluent with the addition of glucose in the SCNR and PCNW phenotypes ($P<0.05$). The highest sperm viability was shown in the RLEYG diluent in the PCNW phenotype ($P<0.05$). The highest abnormality was found in the RLEY and RLEYF diluents in the SCNW, PCNR, and PCNW groups, whereas in the RLEYG group, it was only found in the PCNR group. From the results of this study, it can be concluded that the type of glucose and chicken phenotype influences the quality of post-thawing semen. The best is found in diluents with glucose attachments in the SCNR phenotype.

Key Words: Chicken Semen, Comb, Extender, Medium Supplementation, Freezing

INTRODUCTION

Superior Balitbangtan chickens (KUB) are local indigenous chickens from West Java developed by researchers at the Indonesian Research Institute for Animal Production (IRIAP), Bogor (Bakrie et al. 2021). KUB chickens have high egg production (89.10 eggs/6 months), and males can reach a body weight of 1 kg within 2.5 months and are more resistant to disease (Silalahi et al. 2019). This chicken has been distributed

in Indonesia, including North Sumatra (Hasyim et al. 2020), Jawa Tengah (Hidayah et al. 2019), and Central Sulawesi (Wardi, 2019). According to the Indonesian Agricultural Minister No. 274/Kpts/SR.120/2/2014 (Ditjen PKH 2014) that the phenotypic characteristics of KUB chickens are variable; the feathers of KUB chickens are mostly black (64%), yellow to black, the legs are blackish-grey, with a predominantly single comb (71%), and the rest are pea-shaped.

The phenotype of KUB chickens, such as feather color and comb type reported, influenced semen quality. Phenotypic differences between chickens affect semen characteristics, e.g., comb size (Makhafola et al. 2012). Feather color can also indirectly affect semen quality. Feather colorations strongly correlate with carotenoid metabolism in birds (Weaver et al. 2018). Carotenoids contained in avian plasma can protect cell membrane proteins from oxidative damage (Lucas et al. 2014). Roosters with pale chest feathers had higher levels of oxidative stress, indicated by high levels of malondialdehyde (MDA). MDA was indicated as an indicator of lipid peroxidation, which is believed to cause low sperm motility (Pintus & Ros-Santaella, 2021) and reduce sperm fertility (Thelie et al. 2019). Combs on chickens function as thermoregulation to suppress the incidence of heat stress in chickens. According to (Adedeji et al. 2015), the heat stress index in chickens decreased with the increase in comb size. Chickens with heat stress conditions have more MDA levels (Mujahid et al. 2007), which causes a decrease in motility (Harsha et al. 2021).

Chicken is an animal with a short life span; therefore, the superior genetic material of the KUB chicken must be preserved. According to (Sartika 2016), KUB chickens are selected based on egg production. Therefore the information on semen quality is limited. Genetic material from superior roosters can be conserved to preserve germplasm and genetic diversity in chickens and to be used after the death of the animals (Telnoni, 2016). However, the success of cryopreservation in poultry semen is still low compared to Ruminants. The motility of thawed sperm from cocks varies between 34-40% (Pranay et al. 2018), 27-34% (Mosca et al. 2019), and 40–54% (Askarianzadeh et al. 2018). The morphometry of poultry sperm is a relatively small area. Therefore, the addition of semen diluent may affect the osmotic pressure of the sperm, resulting in damage to the cell membrane (Long 2006).

Biological and biophysical factors affect sperm resistance to freezing, such as membrane permeability, lipid composition, and membrane fluidity (Blesbois 2007; Blesbois et al. 2007). The lipid components in the membrane are divided into three classes, namely phospholipids, cholesterol, and glycolipids (sugary lipids) (Albert et al. 2002). This study used ringer lactate egg yolk as a base extender. Ringer's lactate contains salt, which has a buffering and isotonic effect that maintains sperm motility (Iswati et al. 2018). Egg yolk has been widely used as a poultry semen thinner (Saleh et al. 2022) because it contains low-density lipoprotein that protects sperm (Alkali et al. 2022). The addition of sugar can modulate the fluidity of goat sperm membranes through interactions with the phospholipid bilayer (Aboagla & Terada 2003). The use of sugars (fructose and glucose) in chicken semen diluent for cryopreservation is still limited. Fructose had a better effect than glucose in maintaining the motility of dog sperm which was preserved at five ºC (Ponglowhapan et al. 2004). Using fructose has been reported to maintain the motility of chicken spermatozoa at four ºC for 12 hours, with motility of 63.3%. (Getachew et al. 2015). However, the production of lactic acid from the byproduct of the anaerobic metabolism of fructose is higher than glucose (Li et al. 2017), where the abundance of lactic acid in chicken semen diluent has a toxic effect that causes a decrease in sperm motility (Rochmi & Sofyan 2019).

The success of cryopreservation must be supported by using a cryoprotectant that matches the composition of the semen. In this study, dimethylsulfoxide (DMSO) was used as an intracellular cryoprotectant to prevent the formation of ice crystals in cells. In addition, dimethylsulfoxide has hydrophobic properties that can diffuse through the plasma membrane and replace the phospholipid portion damaged by cryopreservation (Rakha et al. 2016). Although DMSO has benefits in semen freezing, the use of DMSO in chicken semen freezing is also limited. According to research results (Junaedi et al. 2016), using 7% DMSO in chicken semen diluent resulted in the highest quality of PTM and reported (Rakha et al. 2016) the best dose of DMSO for poultry semen diluent is 8%. In this study, the quality of fresh cock semen and the effect of monosaccharides (glucose and fructose) in Ringer lactate egg yolk (RLKT) diluent on the quality of frozen KUB cock semen with different phenotypes are investigated.

MATERIALS AND METHODS

All procedures in this study were approved by the Balitbangtan Animal Ethics Board (KKHB) under No: Balitbangtan/Balitnak/A/03/2021.

Animals

The chickens used were 20 KUB cocks aged ±1.5 years divided into four groups with different phenotypes: Single comb and black or dark brown feather color with feather neck red color (SCNR), Single comb and greenish-black feather color with white feather neck color (SCNW), Pea comb and based black or dark brown feather color with feather neck red color (PCNR) and Pea comb and greenish-black feather color with white feather

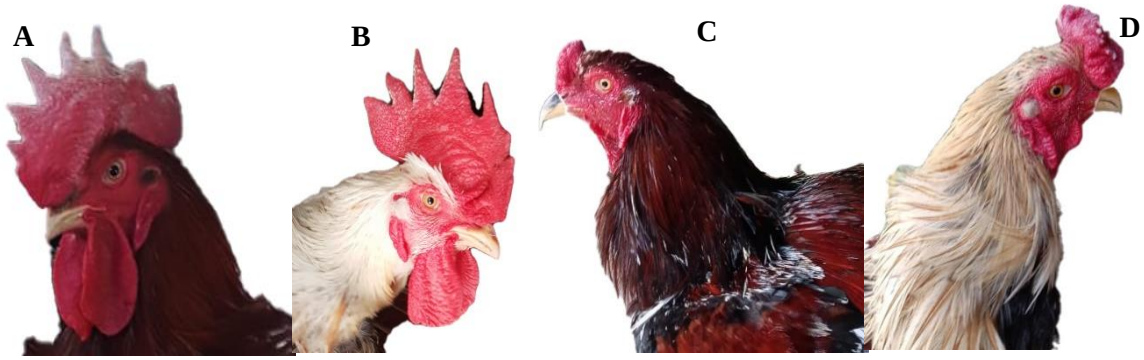

Figure 1. A) Single comb and black or dark brown feather color with feather neck red color (SCNR), B) Single comb and greenish-black feather color with white feather neck color (SCNW), C) Pea comb and based black or dark brown feather color with feather neck red color (PCNR), D) Pea comb and greenish-black feather color with white feather neck color (PCNW)

neck color (PCNW) (Figure 1). The chickens were reared in a cage of 45 cm × 35 cm × 45 cm, received 120 g/head/day and were given water ad libitum.

Diluent preparation

Ringer lactate egg yolk (RLEY) diluent is prepared from 83% Ringer lactate (PT. Widatra Bhakti), 10% egg yolk, and 7% Dimethyl Sulfoxide (DMSO), according to (Junaedi et al. 2016). First, all the ingredients were mixed and homogenized. Then 1000 IU/mL penicillin and 1 mg/mL streptomycin were added to the diluents. The carbohydrates used in this study were 0.6% (w/v) of glucose (RLEY-glucose) and 0.6% (w/v) of fructose (RLEY-F) (Octa et al. 2014).

Semen collection and evaluation

Semen is collected weekly using the abdominal massage method using a 1.5 mL microtube. The massage method is performed continuously on the back of the chicken up to the base of the tail until the chicken shows symptoms of erection (Tarif 2013). Semen from each group was pooled according to the phenotypic group and examined for its quality.

Semen quality was examined macroscopically and microscopically. Macroscopic examinations such as semen volume, pH, color, and consistency were evaluated. Microscopic evaluations included mass movement, sperm motility, viability, abnormality, and sperm concentration. The mass movement was evaluated by dripping semen on the object glass and observed under a light microscope. Assessment is determined using the scoring method (+++/3). Sperm motility was observed by dripping one drop of semen on a glass object, adding 8-10 drops of Ringer's lactate, and then homogenizing. The mixture was then observed under a microscope for five fields of view and expressed in percentages. Observations of viability and abnormalities were carried out simultaneously, mixing one drop of semen and 8-10 eosin nigrosin on a glass object and then preparing a smear. The preparations were then dried on a heated table and observed under a microscope for ten fields of view. Sperm concentration was calculated using a Neubauer chamber with a dilution of 500 times.

The evaluation procedure refers to Arifiantini (2012), adapted for poultry semen. Sperm motility was assessed by diluting semen with lactated Ringer's solution, homogenized, and viewing it under the microscope. Sperm motility was assessed using five fields, and the values were expressed as a percentage. In addition, abnormal sperm and viability were stained with eosin nigrosin. The Neubauer chamber was used to calculate the concentration of sperm—all microscopic evaluations were performed with a binocular microscope (Olympus CX 23).

Semen from each individual per group is pulled and analyzed for motility. Semen with sperm motility of >70% were used in this study and divided into three aliquots diluted with RLEY, RLEYG, and RLEYF, respectively, to achieve a concentration of 200 $\times 10^6$ per mL divided into four straw. The diluted semen was then filled into 0.25 ml mini straws (Minitube, Germany) and kept at equilibrium at 5^{o}C for 2 hours (Junaedi et al. 2016). After equilibration, the straws were placed 6 cm above the surface of the liquid nitrogen for 10 minutes and then stored in a liquid nitrogen container for further evaluation. The frozen semen was analyzed 24 hours after freezing. The method refers to Khaeruddin et al. (2020) with slight modification. Before evaluation, the semen was thawed at 37^{o}C for 30 seconds (Kaheruddin et al. 2020). The evaluation of frozen semen quality was based on sperm motility, viability, morphology, recovery rate, decreased viability and increased abnormality. The sperm motility, viability, and morphology tests were similar to those performed on fresh semen with slight modification. Recovery rate decreased viability and increased abnormality were calculated by comparing the motility of post-thawing sperm and fresh semen.

Data analysis

This study used a factorial, completely randomized design with two factors: the first factor is a chicken phenotype, and the second factor is semen diluent. All procedures were repeated five times. Sperm motility, viability, and morphology data are reported as mean ±SEM. The research data were analyzed using IBM SPSS Statistics 25.

RESULTS AND DISCUSSION

Quality of fresh KUB semen quality with different phenotype

The characteristics of fresh semen of KUB chickens based on the phenotype are shown in table 1. The semen volume of the SCNR group was lower than that of the SCNW and PCNR groups (P<0.05) but not different from PCNW (P>0.05). The characteristics of fresh semen of KUB chickens based on the phenotype are shown in table 1. The semen volume of the SCNR group was lower than that of the SCNW and PCNR groups (P<0.05) but not different from PCNW (P>0.05). Microscopic quality only sperm motility and ejaculate concentration showed the difference. Sperm motility in the SCNR group was lower than SCNW and PCNR (P<0.05) but not different from PCNW (P<0.05). The concentration of sperm per ejaculate in the SCNR group showed the lowest number among all groups (P<0.05), but among the other three groups, there was no significant difference (P>0.05).

Table 2 shows that sperm with diluents RLEY and RLEYF did not differ in each phenotype (P>0.05), while semen with added glucose (RLEYG) showed differences where the SCNR phenotype showed the highest motility and did not differ from PCNW (P>0.05) but differed from that of PCNW (P>0.05). SCNW and PCNR (P<0.05). Semen in the SCNR and PCNW phenotype groups were different in each diluent (P<0.05), while the group only with glucose added (RLEYG) showed the highest motility (P<0.05), but in the PCNR group, the semen in RLEY diluent was lower than RLEYF and RLEYG (P<0.05). Overall, sperm quality after freezing in this study was generally lower than in previous studies. For example, KUB chicken semen diluted in RLEY+ genistein 10 M showed sperm motility of 43% after freezing (Arif 2020). It was also reported by Junaedi et al. (2016) that free-range chicken semen cryopreserved using RLEY showed a post-thaw motility (PTM) value of 37.22% with a viability of 54.47%. Sentul chicken semen in RLEY+7% glycerol provides 30.18% PTM and 47.07% sperm viability. In Telnoni (2016), it was reported that the PTM of Sentul Kampung (SK) Kedu chicken in RLEY+10% DMSO was about 40.83%. The differences between this result and previous studies showed differences in the resistance of chicken sperm to freezing and the compatibility between the semen composition of individual chickens and the diluent. Our preliminary studies using dimethylformamide (DMF) 7% as a cryoprotectant in

Table 1. Fresh KUB rooster semen quality

Variable	Phenotypic			
	SCNR	SCNW	PCNR	PCNW
Macroscopic				
Semen volume (mL)	0.48±0.02^{a}	0.61±0.02^{b}	0.66±0.04^{b}	0.54±0.10^{ab}
pH	6.76±0.03^{a}	6.80±0.03^{b}	6.71±0.01^{a}	6.70±0.00^{a}
Consistency	thick	thick	thick	thick
Color	Milky white	Milky white	Milky white	Milky white
Microscopic				
Mass movement	2.40±0.15	2.30±0.12	2.70±0.10	2.45±0.13
Sperm Motility (%)	75.00±2.29^{a}	79.50±0.88^{b}	80.50±0.71^{b}	76.00±1.57^{ab}
Sperm Viability (%)	88.46±2.63	89.43±1.95	88.96±2.02	89.72±1.89
Sperm abnormalities (%)	14.59±2.29	16.40±1.81	16.18±2.58	16.66±2.00
Sperm concentration (x 10^{6}/Ejaculate)	737.54±90.53^{a}	903.23±101.27^{b}	880.19±77.24^{b}	923.70±70.23^{b}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. $^{a-c}$ superscripts on the same column show a significant difference extender, and $^{A-C}$ superscripts on the same line show a significant difference phenotype in P<0.05

RLEY diluent were also less suitable for KUB chicken semen. KUB chicken results from six generations of kampung chicken whose egg production was selected according to specific criteria and is probably not compatible with RLEY diluent; therefore, it is necessary to use it with other types of diluents.

The lack of success in chicken semen cryopreservation is also caused by the small cytoplasm, which causes a lack of cryoprotectant movement (Svoradova et al. 2021). The freezing process can reduce sperm quality caused by the formation of ice crystals, cold shock, and lipid peroxidation. Therefore, freezing can reduce the percentage of live sperm and increase abnormal sperm (Siudzińska & Ukaszewicz 2008); this can be seen in Tables 6 and 7, where the decrease in post-thawing viability reached 43-60%, and an increase in abnormality reached 19-32%. The freezing process can also cause the formation of ROS, which can disrupt the stability of the sperm membrane. Poultry sperm are susceptible to forming reactive oxygen species (ROS) due to freezing (Rezaie et al. 2021). The production of ROS in sperm takes place in the membrane and mitochondria. An increase in dysfunctional sperm correlates with ROS production, decreasing mitochondrial function (Dutta et al. 2019). High ROS production can damage the plasma and acrosome membranes, leading to infertile sperm (Khan et al. 2021). Reactive oxygen species can be suppressed in the presence of antioxidants, either from seminal plasma or in addition to diluents.

The percentage of viability is shown in table 3, where the difference in viability is only shown in the RLEY diluent, namely the SCNR phenotype was not different from that of SCNW ($P<0.05$) and was higher than PCNR and PCNW ($P>0.05$). The phenotype of SCNW was also not different from that of PCNW ($P>0.05$) but higher than that of PCNR ($P<0.05$). The SCNR group did not show any difference in viability in each diluent. Semen from the RLEY group had the lowest viability than RLEYF and REYG in the SCNW and PCNR groups ($P<0.05$), but there was no difference in viability in the group with the addition of carbohydrates ($P>0.05$). The PCNW group showed differences in the viability of each diluent group, where sperm in the RLEY diluent had the lowest viability and RLEYG had the highest viability ($P<0.05$). The decrease in viability can be seen in table 6. Table 6 shows that only the RLEY diluent showed significant differences in each phenotype. The percentage decrease in viability in the SCNR phenotype was lower than that of PCNR and PCNW but not different from SCNW ($P<0.05$). SCNW phenotype had a percentage decrease in viability that was not different from PCNW ($P>0.05$) but different from PCNR, while PCNW did not differ from PCNR. Differences in decreased viability between diluents in phenotype were not shown in the SCNR phenotype ($P>0.05$). The PCNW phenotype group showed differences in the decrease in viability of each diluent, where the RLEYG diluent had the lowest percentage, followed by RLEYF, and the highest was RLEY ($P<0.05$). Decreased viability is also

Table 2. The motility of KUB Frozen-thawed semen in three different diluent and different phenotype

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	11.60±1.33^{a}	8.96±1.50^{a}	10.56±1.56^{a}	10.64±1.79^{a}
RLEYF	16.88±1.72^{b}	14.00±1.58^{a}	18.72±1.54^{b}	16.60±1.72^{b}
RLEYG	29.80±1.24^{Ac}	20.80±2.21^{Bb}	22.20±1.75^{Bb}	25.40±1.11^{ABc}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. a-c superscripts on the same column show a significant difference extender. A-C superscripts on the same line show a significant difference phenotype in $P<0.05$

Table 3. Viability of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	38.74±2.71^{A}	34.33±2.54^{ABa}	25.45±1.78^{Ca}	29.31±2.70^{Ba}
RLEYF	39.56±2.90	39.25±2.38^{ab}	37.48±2.41^{b}	36.55±2.41^{b}
RLEYG	44.94±2.01	42.58±2.41^{b}	41.87±2.27^{b}	46.30±1.89^{c}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. a-c superscripts on the same column show a significant difference extender, and A-C superscripts on the same line show a significant difference phenotype in $P<0.05$

Table 4. The recovery rate of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	15.47±1.78^{a}	13.28±1.96^{a}	11.13±1.86^{a}	14.00±2.36^{a}
RLEYF	22.51±2.30^{b}	23.55±1.94^{b}	17.39±1.96^{a}	21.84±2.27^{b}
RLEYG	39.73±1.65^{Ac}	27.93±2.21^{Bb}	25.84±2.75^{Bb}	33.41±1.47^{Bc}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. $^{a\text{-}c}$ superscripts on the same column show a significant difference extender, and $^{A\text{-}C}$ superscripts on the same line show a significant difference phenotype in P<0.05

Table 5. Abnormality of KUB frozen-thawed semen in different extender and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	34.23±2.91^{A}	47.78±4.73^{Ba}	44.31±4.30$^{A\ Ba}$	43.71±2.02^{AB}
RLEYF	40.43±1.56^{A}	41.75±1.99$^{A\ ab}$	48.49±2.01^{Ba}	46.49±2.66^{AB}
RLEYG	39.89±1.63^{A}	35.54±1.57$^{A\ b}$	34.24±2.91$^{A\ b}$	47.98±1.36^{B}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color, PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. $^{a\text{-}c}$ superscripts on the same column show a significant difference extender, and $^{A\text{-}C}$ superscripts on the same line show a significant difference phenotype in P<0.05

defined as an increase in the number of dead sperm, which decreases motility. Dead sperm produce lactic acid, increasing the degree of acidity in the media (Iswati et al. 2018). Lactic acid triggers cytoplasmic acidity, which affects the immotile flagellum (Matsuzaki et al. 2015).

The freezing capability of semen derived from each phenotype diluted using RLEY, RLEYF, and RLEYG is shown in table 4. The difference in the percentage recovery rate (RR) of the diluent group in phenotype was only shown in the group with added glucose (RLEYG), where SCNR had the highest RR compared to SCNW, PCNR, and PCNW. However, the three phenotypes were not different (P>0.05). The recovery rate compares post-thawing motility to fresh semen motility and describes the cryodamage index (Buffone et al. 2012). Damage during cryopreservation is caused by dramatic changes in temperature, osmotic pressure, toxic effects of cryoprotectant, and generation of ROS due to oxidative stress (Kumar et al. 2021). Therefore cryopreservation also causes cell death caused by the formation of intracellular ice crystals that can penetrate the membrane and affect cell function (Oberoi et al. 2014).

Abnormalities can cause decreasing motility, and their increase in the treatment is also high. The diluent did not affect abnormalities in the SCNR and PCNW phenotype groups (P>0.05). Meanwhile, semen with added glucose (RLEYG) had the lowest abnormality in SCNW and PCNR phenotypes (P<0.05). Sperm without added carbohydrates (RLEY) had the lowest abnormality in the SCNR phenotype, although not different with PCNR and PCNW (P>0.05) but different with SCNW (P<0.05). Sperm abnormalities with added glucose (RLEYG) in the SCNR, SCNW, and PCNR groups did not show a different percentage (P>0.05) but were lower than in the PCNW group (P<0.05). Sperm abnormality is an essential factor in determining sperm fertility, one of which is the excessive production of ROS (Agarwal et al. 2014). Cryopreservation causes an increase in the production of ROS, which stimulates lipid peroxidation, thereby reducing the integrity of the membrane so that the deformation of cell organelles and protein denaturation cannot be avoided (Partyka et al. 2012). Therefore, in the semen freezing process, it is necessary to add cryoprotectants to minimize damage to sperm. Cryoprotectant is required for the cryopreservation of sperm. There are two types of cryoprotectants: extracellular and intracellular. Extracellular cryoprotectants protect the cell membrane from the osmotic pressure created by freezing; one of these agents is sugar (Darsini et al. 2019). Osmotic stress can cause structural changes in sperm, altering sperm morphology (Yeste et al. 2017). After thawing, the quality of the frozen semen decreases significantly in three different diluents. On the other hand, after sperm disposition at the female genital tract, the avian sperm is stored in the sperm storage tubule (SST) before fertilization.

Decreased sperm viability (DV) showed in Table 6, where the percentage decrease in viability in the SCNR phenotype showed no significant difference in each

diluent. The percentage of sperm viability in the RLEY diluent group showed the highest decrease in sperm viability in each phenotype ($P<0.05$), while the RLEYG diluent only had the lowest percentage in the PCNW phenotype group ($P<0.05$). The different decrease in viability in each phenotype group was only shown by the RLEY diluent, where the SCNR phenotype had the lowest DV compared to the other phenotypes ($P<0.05$). In contrast, the two diluents with added sugar showed no difference in each phenotypic group ($P>0.05$). The RLEYG diluent showed the lowest DV percentage compared to the other two diluents, indicating that sugar can more maintain sperm viability than fructose or without sugar.

Sperm motility and viability are closely related to mitochondrial activity. High mitochondrial activity is positively correlated with the rate of cell metabolism and leads to a high survival rate (Choi et al. 2013). The opening of the mitochondrial pore by freezing causes the release of pro-apoptotic in the cytoplasm and thus promotes cell degradation (Fang et al. 2020), which affects sperm motility (Feyzi et al. 2018). According to (Treulen et al. 2018). Reactive oxygen species that cause pathological calcium excess in somatic cell mitochondria set off the mitochondrial permeability transition (MPT), resulting in mitochondrial malfunction and cell death, directly involved in sperm cell destruction. Sperm motility in chickens correlates with glucose uptake used for sperm motility. These results are based on the study reported by (McLean et al. 1997) that single-combed chickens absorb glucose better than rosy-combed chickens. According to McGary et al. (2002), broad-combed chickens have better fertility than small-combed chickens. Table 7 shows the abnormality (IA) increase in each diluent in each phenotypic group. The increase in abnormality based on the diluents was only seen differently in the SCNW, and PCNR phenotypes, where the RLEY diluent showed the highest percentage and RLEYG had the lowest percentage ($P<0.05$). The highest percentage of IA in the RLEY diluent was from the SCNW phenotype, and the lowest was from the SCNR phenotype ($P<0.05$). The increase in abnormalities in the RLEYF diluent of the PCNR phenotype was not significantly different from that of the PCNW phenotype but was higher than the SCNR and SCNW groups ($P<0.05$). The percentage increase in abnormalities in sperm diluted using RLEYG showed no difference with the SCNW and PCNW groups ($P>0.05$) but was higher than the PCNR groups ($P<0.05$). Overall, the percentage of IA in the SCNR phenotype group was only around 19-25%. It has been described above that single-combed chickens can absorb glucose better, so this may be one of the reasons the results in the SCNR group are better than the other phenotypes.

In this study, the chickens of the SCNR phenotype showed better sperm motility than sperm from other phenotypic groups. SCNR is thought to contain carotenoids that act as antioxidants against free radicals during freezing. Carotenoids strongly correlate with plumage coloration in birds (Weaver et al. 2018). Carotenoids are non-enzymatic antioxidants that protect sperm from oxidative stress (Triques et al. 2019). Carotenoids are found in the inner membrane of the mitochondria, which have closely related to the function of the mitochondria as a site for aerobic cell respiration (Hill et al. 2019). The carotenoid content, which is thought to be present in the SCNR phenotype, may protect sperm from free radical damage. It has the lowest abnormality compared to the other three phenotypes. Low sperm abnormalities were negatively correlated with PTM and sperm viability. This correlation indicates low abnormal morphology leading to PTM and high viability in sperm.

Morphological defects or types of abnormalities in this study refer to (Feyisa et al. 2018), who reported that there were 11 types of abnormalities in chicken sperm, namely coiled head, bent head, broken head, knotted head, detached head, broken midpiece, bent tail, knotted tail, broken tail, coiled tail, detached tail. The results showed that a broken midpiece (BM) was the most common morphological defect. The percentage of sperm BM of each diluent in the phenotype showed that only the RLEYG diluent had differences. The SCNW phenotype group had the lowest percentage that differed from PCNW ($P<0.05$) but did not differ from SCNR and PCNR ($P>0.05$). Broken midpiece sperm of each phenotype in the diluents showed that the SCNW group had different percentages in each diluent. The sperm group in the REG diluent had the lowest percentage compared to RLEY and RLEYF ($P<0.05$), while in both diluents, there was no significant difference ($P>0.05$). It is well known that the mitochondria, which are the site of ATP metabolism, are located in the midpiece of the sperm. Therefore, damage to the midpiece area is believed to decrease sperm motility. The results of ATP hydrolysis in the mitochondria can diffuse into the flagella by activating the dynein arm on the microtubules so that there is a movement of the flagella (Gwo & Arnold 1992), which explains the reason PTM in this study is very low.

The results showed that semen in RLEYG diluent was better than RLEYF and RLEY. Sperm requires energy to move and reach the egg in the female reproductive tract; this process involves ATP to maintain flagellar movement through glucose metabolism or glycolysis. In semen diluents, glucose has two primary functions: energy supply and cryoprotectant agent. Glucose could directly decompose to give energy to sperm, but fructose had to be turned into glucose before it could supply energy (Tang et al. 2021). The glycolysis process is supported by a transporter protein that transports glucose into the sperm membrane in the midpiece called glucose transport (GLUT) (Setiawan et al. 2020).

Table 6. Decreased viability (DV) of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	49.72±2.70^{A}	55.09±2.54^{ABa}	63.50±1.78^{Ca}	60.41±2.69^{BCa}
RLEYF	48.90±2.90	50.17±2.38^{ab}	51.47±2.35^{b}	53.17±2.41^{b}
RLEYG	43.51±2.01	46.84±2.41^{b}	47.09±2.27^{b}	43.42±1.89^{c}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color and PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. $^{a-c}$ superscripts on the same column show a significant difference extender, and $^{A-C}$ superscripts on the same line show a significant difference phenotype in P<0.05

Table 7. Increased abnormality of KUB frozen-thawed semen in different extenders and phenotypes

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	19.65±2.92^{A}	31.38±4.73^{Ba}	28.14±4.30^{ABa}	27.05±2.02^{AB}
RLEYF	25.84±1.56 ^{A}	25.34±1.10 $^{A\ ab}$	32.31±2.02^{Ba}	29.83±2.66^{AB}
RLEYG	25.30±1.62^{AC}	19.14±1.57^{ABb}	18.06±2.91^{Bb}	31.33±2.37^{C}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color and PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. $^{a-c}$ superscripts on the same column show a significant difference extender, and $^{A-C}$ superscripts on the same line show a significant difference phenotype in P<0.05

Table 8. Broken midpiece in KUB Post Thawing chicken sperm in different diluents and phenotypes.

Extender	Phenotype±(SEM)			
	SCNR	SCNW	PCNR	PCNW
RLEY	37.75±3.28	37.03±3.27^{a}	34.87±1.95	31.24±3.04
RLEYF	34.90±2.99	36.31±1.54^{a}	39.19±2.24	35.71±2.03
RLEYG	32.43±2.14^{AB}	28.23±2.53^{Ab}	34.15±1.94^{AB}	37.81±2.28^{A}

SCNR= Single comb and based black or dark brown feather color with feather neck red color, SCNW= Single comb and based greeny black feather color with feather neck white color, PCNR= Pea comb and based black or dark brown feather color with feather neck red color and PCNW= Pea comb and based greeny black feather color with feather neck white color. RLEY= Ringer lactate egg yolk, RLEYF= Ringer lactate egg yolk fructose, RLEYG= Ringer lactate egg yolk glucose. $^{a-c}$ superscripts on the same column show a significant difference extender, and $^{A-C}$ superscripts on the same line show a significant difference phenotype in P<0.05

Glucose is transported into the cell by GLUT, and protons will leave the cell. The release of protons from the cell leads to intracellular alkalinization and promotes flagella movement (Mannowetz et al. 2012). In addition, glucose has an excellent cryoprotective effect on sperm by interacting with the plasma membranes of sperm, as reported in wild boar sperm (Gómez-Fernández et al. 2012).

Comb growth is controlled by androgens, which are known to regulate spermatogenesis (Yamamoto, 2016). Comb growth is closely related to testosterone and aggressiveness in poultry (Mukhtar & Khan2012). The stimulation of intracellular fluid generation by fibroblasts in the comb and dewlap by testosterone and dihydrotestosterone is known to affect the growth of these tissues (Leão et al. 2017). The comb is located on the head and functions for thermoregulation (Iyasere et al. 2021), so a large comb provides better temperature regulation than smaller combs. However, chickens do not have sweat glands, so their heat dissipation system is not optimal.

For this reason, chickens are more susceptible to heat stress. The optimal body temperature of chickens is between 39.9 - 41ºC. A rise in body temperature can lead to death in chickens (Ramadiani et al. 2021). In addition, birds exposed to temperatures above their thermoneutral zone develop respiratory alkalosis, which stimulates the hypothalamic-pituitary-adrenal axis to produce more cortisol, corticosterone, catecholamines, glucocorticoids, and adrenocorticotropin, which harms their physiology, intestinal function, and reproductive function (Kumar et al. 2021). Heat stress also reduces carbohydrate absorption by decreasing glucose transporter concentrations and amino acid absorption (Goel et al. 2021), resulting in

weight loss and reproductive mechanisms, which explains that single comb and black or dark brown feather color with red feather neck color (SCNR) has better-post-thawing sperm quality compared to other groups.

As described above, sugar can be an extracellular cryoprotectant that can protect spermatozoa from damage caused by cryopreservation. The plasma membrane is covered by glycocalyx sugars, which can bind with lipids and proteins that protect sperm from damage (Herdis et al. 2019). In addition, sugar can be immune-protective, preventing antigenicity during transport in SST (Peláez et al. 2011). Therefore, adding sugar to the semen diluent can replace the carbohydrate component in the plasma membrane, which is damaged by freezing. Moreover, glucose can inhibit the performance of 2-deoxyglucose (DOG), which can hinder the glycolysis process, while the presence of fructose is insufficient to prevent the performance of 2-deoxyglucose (DOG). 2-deoxyglucose is a glucose analog that can inhibit the role of hexokinase, one of the enzymes regulating glycolysis (Pasupuleti 2007).

Sperm abnormalities after thawing showed a significant effect of freezing on chicken sperm. Cryopreservation can increase osmotic pressure so that the plasma membranes of the cell can be damaged (Miranda et al. 2018). Poultry semen is very sensitive to freezing due to its different morphology from mammalian sperm. The smaller surface of the sperm makes the poultry sperm vulnerable to osmotic pressure and freezing (Long 2006). Single-comb chicken sperm can absorb glucose better than pea-comb chicken. This result could explain the lowest sperm abnormality in the RLEYG diluent. Normal sperm have more opportunities to fertilize the egg. Therefore morphology is an essential factor when evaluating sperm. Freezing can cause changes in sperm morphology. These changes are caused by changes in the ultrastructural of sperm due to drastic temperature changes, osmotic pressure, and the formation of crystal ice in sperm (Sharma & Sharma 2020).

Consequently, the crystal ice formed by cryopreservation can lead to membrane structure, integrity, and morphological changes (Darsini et al. 2019). Apart from crystal ice formation, swelling and shrinkage of cells due to water changes and cryoprotectants are also unavoidable in the early stages of freezing, which most cell organelles cannot tolerant (Medeiros et al. 2002; Ozkavukcu et al. 2008). It has been described above that the small surface of the sperm at the time of freezing is susceptible to osmotic pressure. The low results of PTM semen in this study could have several causes. The first possibility is that the diluent used in this study is incompatible with KUB chicken semen. Some of the diluents commonly used in chicken semen include Beltsville poultry semen extender (BPSE) (Telnoni 2016), Tris-Skimmed milk (Bustani & Baiee 2021), Ringer's Lactate Low-Density lipoprotein for chicken or quail (Magfira et al. 2017), and a combination of coconut water, egg yolk, and fructose (Rochmi & Sofyan 2019). Another possibility is that KUB chickens have low freezing capacity. In addition, KUB chickens result from six generations of selection which can lead to changes in semen quality after freezing. Semen quality in F1 to F5 from KUB chicken has not been reported, so there is no comparison of semen quality. However, (Ansah & Buckland 1983) found that the fertile period of frozen semen in selected hens increased in generations F3 to F7 and decreased in generation F8. This result suggests a selection effect on the quality of frozen chicken semen. Factors influencing freezing ability have been widely reported, such as genetics and individual variation within a pedigree (Tesfay et al. 2020); it was reported that genetic differences reflected in the semen volume, pH, motility, viability, and abnormalities of sperm (Furthermore, it was reported that pH was correlated with sperm abnormalities (Natarajamani et al. 2014). These differences in the fresh semen quality allow the quality of frozen semen to be influenced by pedigree or genetics.

CONCLUSIONS

The best motility and recovery were found in the SCNR phenotype group, diluted using added glucose. The PCNW group had high abnormalities in all diluent groups. In this study, abnormalities significantly affect the decrease in the motility of chicken semen. Further research is needed to evaluate the seminal plasma semen content of KUB chicken and also to find the more suitable freezing diluents.

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