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Evaluation of Japanese Quail Egg-Yolk Extender in Cryopreservation of Cockerel Semen

[1]Rantloko Rolly Maapola, [1]Khathutshelo Agree Nephawe, [1,2]Tshimangadzo Lucky Nedambale and [2]Fhulufhelo Vincent Ramukhithi

[1]Department of Animal Science, Tshwane University of Technology Pretoria, Private Bag X 608, 0001, South Africa
[2]Agricultural Research Council, Animal Production, Germplasm, and Reproductive Biotechnologies, Private Bag X2 Irene, South Africa

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Corresponding Author:
Fhulufhelo Vincent Ramukhithi
Agricultural Research Council, Animal Production, Germplasm, and Reproductive Biotechnologies,
Private Bag X2 Irene,
South Africa
Email: Fhuluvi@gmail.com

Abstract: The purpose of this study was to examine the effects of quail egg yolk extenders in the cryopreservation of cockerel semen. Ten Venda cockerels were chosen randomly and semen was collected twice a week with a two-day rest period using an abdominal massage technique. Immediately following semen collection, semen samples were evaluated for microscopic parameters (sperm cell motility, viability, morphology, and abnormalities) as well as macroscopic parameters (sperm cell concentration, semen volume, color, and pH). Afterward, the semen samples were divided into two fractions: One was diluted with Japanese quail egg-yolk extenders at different concentrations (0, 5, 10, 15, and 20%) and one was diluted with chicken egg-yolk (20%). Semen samples were evaluated for sperm cell motility using CASA prior to freezing in a programmable freezer. Immediately following freezing, semen samples were stored at 196°C until thawing. Semen samples were thawed and evaluated for sperm cell motility. The data were analyzed using the general linear model of statistical analysis software 9.4. Both microscopic and macroscopic semen parameters evaluated and used for cryopreservation of Venda cockerels' semen were normal and acceptable. The 10% Japanese quail egg-yolk extender resulted in significantly higher total motility (39.7±2.6%) when compared with the 0, 5, 15, and 20% concentrations, which resulted in 9.0±2.7, 22.9±2.7, 22.1±2.7 and 32.1±2.7% total motility, respectively. Moreover, a 10% Japanese quail egg-yolk extender resulted in total motility of 39.7±2.6%, which was significantly higher than the total motility of 31.7±2.6% obtained when the recommended 20% chicken quail egg-yolk extender used. In conclusion, 10% of Japanese quail egg yolk is a suitable concentration that can be used to cryopreserve cockerel semen.

Keywords: Cryopreservation, Cockerel Semen, Extenders

Introduction

Indigenous breeds such as Venda, Ovambo, Potchefstroom koekoek, and naked neck thrive in harsh environments, are resistant to most local diseases, can endure cold and hot temperatures, and wet or dry conditions, and are relatively inexpensive to raise (Grobbelaar *et al*., 2010). Nevertheless, some of them are not managed well due to a lack of information about their reproductive performance (Manyelo *et al*., 2020). In addition, they are endangered due to crossbreeding and the introduction of exotic breeds. Conservation of these breeds is important in order to protect their diversity because once they become extinct, they cannot be replaced (Ramukhithi, 2016). The main goal of conserving genes is to keep superior and different genes as pure as possible (Mara *et al*., 2013). Due to this, it is critical to employ modern technologies, such as cryopreservation, to help improve the productivity of indigenous chickens (Keim *et al*., 2009).

Cryopreservation is the process of freezing tissues and cells at 196°C. Cryopreservation of cockerel semen remains a challenging obstacle due to the lower sperm cell survival rate following the freeze thawing process. Furthermore, progress has been made in developing semen diluents and cryopreservation procedures for poultry semen (Makhafola *et al*., 2009); however, the survival and motility rates still remain poor. In poultry breeding, the fertility rate is very low when frozen thawed

semen is used in artificial insemination (Getachew, 2016). The cockerel's sperm cell shape is different compared to other mammalian species, which might be playing a critical role in their lower survivability. Therefore, it is important to develop a suitable extender or diluent for preserving the cockerel's semen. Moreover, the poor sperm cell survival rate has been documented to be due to the poor cryopreservation mediums and protocols used (Dhama *et al*., 2014). The addition of chicken egg yolk to semen extenders is thought to reduce the fertilizing potential of cockerel sperm cells but not that of other avian species (Gholami *et al*., 2012). Makhafola *et al*. (2009); Mphaphathi (2011) indicated that the survivability and motility of sperm cells were negatively affected during the cryopreservation and thawing of cockerel semen when chicken egg yolk was used.

Akhter *et al*. (2017) reported that quail egg yolks contain low levels of lipoprotein, which was shown to be more protective during the cryopreservation process and resulted in better sperm cell progression, motility, and viability rates. This is due to the presence of phospholipid, which detoxifies the seminal plasma by grabbing the harmful proteins in it, thereby increasing the viability of sperm cells (Bathgate *et al*., 2006). The use of quail egg-yolk as a poultry semen extender, on the other hand, has not been used in freezing indigenous cockerel semen. The current study was conducted to find whether a quail egg-yolk extender provides cryoprotective advantages over a chicken egg-yolk extender in the cryopreservation of a cockerel's semen.

Materials and Methods

Study Design

Two experiments were conducted in this study. The study design used was 2 × 2 factorial designs. The first experiment was designed to find the suitable quail egg-yolk concentration (0, 5, 10, 15, and 20%) prior to cryopreservation of cockerel semen. In this experiment, sperm cell parameters (motility, viability, morphology, and abnormalities) were evaluated. After successfully obtaining suitable quail egg yolk, the second experiment was designed to compare the effects of quail and chicken egg-yolk based extenders on the cryopreservation of cockerel semen.

Study Location and Animal

The study was conducted at the agricultural research council Irene, Germplasm conservation and reproductive biotechnologies laboratory. Venda cockerels were placed in a well-maintained chicken house. The water and feed (finisher feed) were provided ad libitum. All cockerels were treated according to the guidelines for the care and use of agricultural animals.

Semen Collection and Evaluation

At the age of 26 weeks, cockerels were trained for semen collection for three weeks. Ten cockerels that responded to training were used for semen collection. The semen was collected from each cockerel twice a week with a resting period of three days by using the abdominal massage method (Makhafola *et al*., 2009). Individual ejaculates were collected in a 15 mL tube and placed in a thermos flask maintained at 38°C (Mphaphathi, 2011).

Semen samples were then evaluated for macroscopic and microscopic parameters. Semen color was observed visually and characterized as clear or watery, cloudy, milky, creamy, and thick creamy (Ramukhithi, 2016). Semen volume was measured by reading the measurements on a 15 mL tube and it was recorded in milliliters (Mphaphathi, 2011). Semen pH was measured using litmus paper. Sperm cell concentration was determined using a 6310 spectrophotometer (Jenway, United Kingdom). A computer aided sperm analyzer (Microptic S.L., Barcelona) was used to measure sperm cell motility and velocities. Sperm cell morphology samples were evaluated using a fluorescent microscope (BX51TF) (Olympus, Japan).

Preparation of Extenders

The Japanese quail and chicken egg yolks were collected and placed in egg cartons before preparing the extender. After breaking the eggs, the yolks were kept in a 50 mL tube. Kobidil + extender, 43.8 g, was dissolved in 1 L of purified water and divided into two fractions shown in (Table 1).

Cryopreservation and Thawing of Semen

The cockerel semen was diluted into fraction A and equilibrated at 5°C for 2 h. After the first equilibration, fraction B was added and kept at 5°C for 2 h. The diluted semen sample was loaded into straws (0.25 mL) near the end of the second equilibration period (10 min.), kept at room temperature, and sealed with straw sealing powder.

Table 1: Composition of quail and chicken egg-yolk extender

Extender	Concentration (%) level	Fraction A (Kobidil)	Fraction B (Kobidil + Dimethyl sulfoxide)
Quail egg-yolk	0	100 mL	90 and 10 mL
	5	95 mL	85 and 10 mL
	10	90 mL	80 and 10 mL
	15	85 mL	75 and 10 mL
	20	80 mL	70 and 10 mL
Chicken egg-yolk	20	80 mL	70 and 10 mL

The straws were frozen for 5 min at 20°C Celsius. They were suspended 4-6 cm above liquid nitrogen vapor and plugged into a Styrofoam® container containing liquid nitrogen. Thereafter, the semen straws were stored in a liquid nitrogen tank (-196°C) (Mphaphathi, 2011). The semen straws were thawed at 37°C for 20 sec. Thereafter, semen samples were again analyzed for sperm cell motility parameters using a computer aided sperm analyzer.

Statistical Analysis

Semen parameter data were analyzed using the Generalized Linear Model (GLM) procedure of statistical analysis software 9.4. Simple statistics and the Least Square means (LS-means) were computed and then the Least Significance Difference (LSD) test was used to compare the means between treatments ($p<0.05$).

Results

The macroscopic semen parameters of Venda cockerels are presented in Table 2. The semen parameters of the Venda cockerel were as follows: Semen volume (0.3±0 mL), sperm cell concentration (53.0×10^6/mL), and pH level (6.8 ± 0).

The microscopic semen parameters of Venda cockerels are presented in Table 3. The microscopic sperm cell motility parameters were as follows: Total motility (90.5±1.2%), progressive motility (61.0±2.5%), and non-progressive motility (29.7±1.9%). Venda cockerel sperm cells were 92.8 0.9% live, 7.4± 0.7% dead, 84.7±0.7% normal, and 15.3 0.7% abnormal. Venda cockerel had 4.2±0.2% primary abnormalities, 7.2±0.3% secondary abnormalities, and 4.1±0.2% tertiary abnormalities.

The effect of Japanese quail egg-yolk concentration on Venda cockerel semen following cryopreservation is presented in Table 4. The 10% quail and 20% egg-yolk extenders resulted in similar total motility (39.7±2.7% and 32.1±2.4%), respectively). The quail egg yolk extender at 10% resulted in high ($p<0.05$) progressive motility (13.4±1.4%).

The 10 and 20% quail egg-yolk extenders resulted in similar non-progressive motility (26.3±1.2 and 23.8±1.2%, respectively). The 5 and 15% resulted in similar static sperm cells (77.1±2.2 and 77.9±2.5%), which were higher ($p<0.05$). The 15% quail egg-yolk extender resulted in a high ($p<0.05$) wobble (66.9±1.3%) compared to other concentrations (0, 5, 10, and 20%).

The effect of Japanese quail egg-yolk and chicken egg-yolk based extenders on Venda cockerel semen after cryopreservation is presented in Table 5. In comparison to the 20% chicken egg yolk extender, which had total motility of 31.4±2.6%, progressive motility (13.4±1.4%), non-progressive motility (26.3±1.2%), rapid velocity (1.8±0.2%) and medium velocity (17.2±1.3%), the 10% Japanese quail egg yolk extender had high ($p<0.05$) total motility (39.7±2.7%), progressive motility (13.4±1.4%), rapid velocity (1.8±0.2%) and medium velocity (17.2±1.3%). The 10% quail egg-yolk extender resulted in high ($p<0.05$) curvilinear (64.5±1.8 μm/s) compared to the 20% chicken egg-yolk extender (60.0±0.9 μm/s). The 10% Japanese quail egg-yolk extender resulted in a high ($p<0.05$) average path velocity (38.54±1.5 μm/s) compared to the 20% chicken egg-yolk extender (35.98±0.7 μm/s).

Table 2: Macroscopic semen parameters of Venda cockerel

Semen parameters	Mean ± SE
Semen volume (mL)	0.3±0
Sperm cell concentration ($\times 10^6$/mL)	53.0±0
Semen pH	6.8±0

Table 3: Microscopic semen parameters of Venda cockerel

Sperm cell parameters			Mean ± SE
Motility	Progression (%)	Total motility	90.5±1.2
		Progressive motility	61.0±2.5
		Non-progressive motility	29.7±1.9
		Static	10.1±1.6
	Velocity (%)	Rapid	26.2±2.9
		Medium	51.1±1.7
		Slow	13.3±1.3
	Average values parameters of velocity	Curvilinear (μm/s)	106.5±3.4
		Straight line velocity (μm/s)	38.3±1.4
		Average path velocity (μm/s)	59.3±2.3
		Linearity (%)	37.2±0.9
		Straightness (%)	60.6±0.7
		Wobble (%)	58.2±0.8
Viability (%)	Live		92.6±0.7
	Dead		7.4±0.7
Morphology (%)	Normal		84.7±0.7
	Abnormal		15.3±0.7
Abnormalities (%)	Primary		4.0±0.2
	Secondary		7.2±0.3
	Tertiary		4.1±0.2

Table 4: The effect of Japanese quail egg-yolk concentration on Venda cockerel semen following cryopreservation

Sperm cell parameters			0% QEY	5% QEY	10% QEY	15% QEY	20% QEY
Motility	Progression (%)	Total motility	9.0±2.7^{d}	22.7±2.0^{c}	39.7±2.3^{a}	22.1±2.5^{c}	32.1±2.7^{ab}
		Progressive motility	2.2±1.3^{c}	6.5±1.2^{b}	13.4±1.4^{a}	6.3±1.6^{b}	8.3±1.7^{b}
		Non-progressive motility	6.8±1.8^{c}	16.4±1.4^{b}	26.3±1.2^{a}	15.8±1.7^{b}	23.8±1.2^{a}
		Static	91.0±2.3^{a}	77.1±2.2^{b}	60.3±2.7^{c}	77.9±2.5^{b}	67.9±2.8^{bc}
	Velocity (%)	Rapid	0.3±0.3^{b}	1.6±0.5^{a}	1.8±0.2^{a}	0.9±0.4^{a}	1.2±0.7^{a}
		Medium	3.1±1.4^{c}	8.2±1.7^{b}	17.2±1.3^{a}	7.9±1.6^{b}	11.8±1.3^{b}
		Slow	5.5±1.2^{c}	13.1±1.2^{b}	20.8±1.2^{a}	13.2±1.2^{b}	19.1±1.2^{b}
	Average values of velocity parameters	Curvilinear (µm/s)	61.1±1.7^{ab}	63.4±1.2^{a}	64.5±1.8^{a}	59.3±1.3^{b}	61.1±1.7^{ab}
		Straight line velocity (µm/s)					
		Average path velocity (µm/s)	23.2±1.3	23.4±1.6	24.8±1.2	25.5±1.4	22.1±1.2
			36.6±1.2	34.8±1.3	38.5±1.5	38.4±1.4	36.6±1.6
		Linearity (%)	38.7±1.5^{b}	37.8±1.4^{b}	40.4±1.7^{b}	44.7±1.2^{a}	38.7±1.3^{b}
		Straightness (%)	59.2±1.1^{b}	58.9±1.3^{b}	61.6±1.4^{a}	63.4±1.1^{a}	59.1±1.2^{b}
		Wobble (%)	60.4±1.2^{b}	60.4±1.2^{b}	61.8±1.4^{b}	66.9±1.3^{a}	62.6±1.6^{b}

$^{a, b, c}$ Values with different superscripts within the same row differ significantly ($p<0.05$). QEY= quail egg-yolk

Table 5: Effect of Japanese quail egg-yolk and chicken egg-yolk based extender on Venda cockerel semen after cryopreservation

Sperm cell parameters			10% QEY	20% CEY
Motility	Progression (%)	Total motility	39.7±2.7^{a}	31.7±2.6^{b}
		Progressive motility	13.4±1.4^{a}	8.0±1.1^{b}
		Non-progressive motility	26.3±1.2^{a}	23.4±1.6^{b}
		Static	60.3±2.7^{a}	68.7±2.6^{b}
	Velocity (%)	Rapid	1.8±0.2^{a}	0.9±0.2^{b}
		Medium	17.2±1.3^{a}	11.8±1.4^{b}
		Slow	20.8±1.2	19.4±1.2
	Average values parameters of velocity	curvilinear (µm/s)		
		Straight line velocity (µm/s)	64.5±1.8^{a}	60.0±0.9^{b}
			24.8±1.2	22.8±0.8
		Average path velocity (µm/s)	38.5±1.5^{a}	36.0±0.7^{b}
		Linearity (%)	40.4±1.7	40.1±1.3
		Straightness (%)	61.6±1.4	61.1±1.1
		Wobble (%)	61.8±1.4	62.0±0.9

$^{a, b, c}$Values with different superscripts within the same row differ significantly ($p<0.05$). QEY = Quail Egg-Yolk, CEY= Chicken Egg-Yolk

Discussion

In this study, Venda cockerels had a semen volume of 0.3 mL, which was comparable to the Venda cockerels stated by Mphaphathi (2011). However, the semen volume in this study was higher compared to the semen volume of 0.2 mL in Venda cockerels (Molekwa and Umesiobi, 2009) and white crested black Polish cockerels (Siudzińska and Łukaszewicz, 2008). On the other hand, the semen volume of the current study was lower compared to Arabic cockerels' semen volume (0.4 mL) (Hermiz *et al*., 2016). The difference between the current and previous studies might be due to different breeds, environments, ages, and feeds.

In this study, the sperm cell concentration of Venda cockerels was 53.0×10^6/mL. This is not comparable to those reported in the same breed (678×10^6/mL) by Mphaphathi (2011). Noteworthy, several authors have reported sperm cell concentrations of 4.5×10^6/mL in Arabian indigenous chickens (Hermiz *et al*., 2016) and 4.6×10^6/mL in black Minorca and white crested black polish indigenous chickens (Siudzińska and Łukaszewicz, 2008).

In this study, the semen pH was 6.8±0 which was similar to the results reported by Mphaphathi (2011) using Venda chickens (6.9±0.4). However, it was lower compared to the semen pH of 7.71 in Cezere indigenous chickens (Tuncer *et al*., 2008) and 7.7 in naked neck chickens (Makhafola *et al*., 2009). The difference between the results found in the present and previous studies is due to age and season (Mphaphathi, 2011).

In the present study, the average sperm cell motility was 90.6% which was in agreement with the results found by Mphaphathi (2011) (91.8%) when the same Venda chicken breed was used. However, Hermiz *et al*. (2016) reported low sperm cell motility of 75.6% in Arabic chickens. Therefore, the sperm cells found in the present study were good and it was reported that the suitable percentage of sperm cell motility in fresh semen, which is preferable before the freezing process, is >70% (Ashrafi *et al*., 2011).

In the current study's average normal sperm cells (92.5%) were similar to the results (92%) reported by Tselutin *et al*. (1999), Venda (90%) by Mphaphathi (2011) and 91.35% in Arabic cockerels (Hermiz *et al*., 2016). However, it was higher when compared to white-crested black Polish normal sperm cells (70.5%) (Siudzińska and Łukaszewicz, 2008) and naked neck (69.9%) normal sperm cells (Makhafola *et al*., 2009).

In this study, the sperm cell abnormalities were 15.3% (4.0±0.2% primary, 7.2±0.3% secondary, and 4.2±0.2%

tertiary) and were higher compared to the results reported by Mphaphathi (2011) (4.6%) using the same breed. It is believed that sperm cell abnormalities come from weak motility. The difference between the results might be due to the season and environment.

In the present study, sperm cell motility after thawing for different concentrations of quail egg yolk extenders was 0% (9.0±2.7%), 5% (22.7±2.0%), 10% (39.7±2.3%), 15% (22.1±2.5%) and 20% (32.1±2.7%) respectively. Following thawing, the 10% quail egg yolk produced higher sperm cell motility (39.7%). Moreover, the results were similar to those reported by Akhter *et al*. (2017), where 10% quail egg yolk resulted in high sperm cell motility (35.6%) after freezing when Poitou Jackass (Equine) was used. Seshoka *et al*. (2016) reported a good sperm cell motility rate (92.6%) after freezing in Nguni cattle using 10% quail egg yolk. On the other side, Kulaksız *et al*. (2010) reported low sperm cell motility (26.6%) using 15% quail egg yolk in ram frozen thawed semen, which is similar to the current results. This is not surprising, as it has been reported that supplementation of 15-20% of the Japanese quail egg yolk can be toxic to the semen due to the high levels of substrate available for the formation of hydrogen peroxide (Akhter *et al*., 2017). Furthermore, lower concentration has a good advantage on sperm cells during cryopreservation because it contains fewer cryoprotectant antagonists such as yolk granules, calcium, progesterone, and low density, which are thought to compromise sperm cell freezability (Akhter *et al*., 2017).

When the best Japanese quail (10%) and chicken (20%) egg-yolk extenders' concentrations were compared. Japanese quail extender (10%) increased sperm cell motility by 39.7%, while chicken egg yolk (20%) resulted in sperm cell motility of 31.4%. This could be because quail egg yolks contain more phosphatidylcholine, less Phosphatidylethanolamine, and a lower ratio of polyunsaturated fatty acids to saturated acid, all of which protect sperm cells from cold shock (Moussa *et al*., 2002; Akhter *et al*., 2017). Therefore, this makes quail egg yolk a better cryoprotectant during cryopreservation when compared to chicken egg yolk. The minimum requirement of sperm cell motility after freezing needed to perform artificial insemination is >30%. There has been a lot of research done to find the best extender for freezing cockerel semen and the following frozen-thawed sperm cell motility was reported at 22.7, 45, 25.71 and 29.06%, respectively (Shahverdi *et al*., 2015; Rakha *et al*., 2018; Malik *et al*., 2019; Khairuddin *et al*., 2019). In recent studies, 47.30 and 41% sperm cell motility were obtained following the freezing of chicken semen when soya bean nanoparticles and DMA extender were used (Sun *et al*., 2021; Tang *et al*., 2021) which is a positive result. However, there is still a gap, and more research needs to be done.

Conclusion

Following cryopreservation of cockerel semen using Japanese quail egg yolk, 10% performed better than the other treatments (0, 5, 15, and 20%). Moreover, 10% Japanese quail egg yolk resulted in high sperm cell motility after freezing when compared with 20% chicken egg yolk (control). In conclusion, 10% of Japanese quail egg yolks can be used as an alternative to cryopreserve cockerel semen.

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Author's Contributions

All authors equally contributed to this study.

Conflict of interest

All authors have received and approved the final version of the manuscript. Our submission has no conflict of interest.

Ethics

The study ethics were evaluated and approved by the agricultural research council ethics committee and the Tshwane University of technology ethics committee (reference APIEC17/23).

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