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SEMEN CHARACTERISTICS OF PARAOAKAN NATIVE CHICKEN BEFORE AND AFTER CRYOPRESERVATION USING GLYCEROL OR DIMETHYL SULFOXIDE IN AU BASED SEMEN EXTENDER

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ABSTRACT

Chicken semen cryopreservation is an essential tool for programs of genetic diversity management and conservation of endangered breed lines particularly the Philippine native chicken. However, the method still needs improvement for it to be applied in a wide variety of environments and breeds. This study compared the effects of two permeating cryoprotectants (dimethyl sulfoxide, also called DMSO, and glycerol) during the freezing of semen from Paraoakan native chickens, grouped into either good or poor batches based on their CASA-evaluated initial sperm motility and by adopting a reported freezing protocol for avian using liquid nitrogen. Here, the two cryoprotectants (DMSO and glycerol) were tested for their post-thawing efficiency. The cryopreservation method was done using 0.5 mL straws with AU as its diluent. Different semen parameters (i.e., motility, viability, and progressive motility) were evaluated before and after cryopreservation. Results showed that glycerol is a better cryoprotectant in terms of post-thawing motility and viability ($P<0.005$). Moreover, glycerol and DMSO exhibited no difference ($P<0.005$) in terms of progressive sperm motility after thawing. Additionally, our results show that motility has a monotonic direct relationship with viability ($P<0.005$).

Key words: cryopreservation, DMSO, glycerol, Paraoakan, Philippine native chicken

INTRODUCTION

The Philippine native chickens are gaining popularity in many households due to their unique flavor, protein content, and nutritious meat. These birds are predominantly raised under a free-range system in most local areas while some farmers raise them under semi-confinement (Mananghaya, 2017). In addition, most farmers prefer to raise native chickens over the exotic breeds because of their relatively low input, inherent ability to survive under harsh local environmental conditions, and their ability to reproduce even under minimal care and marginal management (Lambio, 2000). Native chickens are claimed to be relatively resistant to many common local poultry diseases and are good foragers who can thrive on

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farm by-products. However, several factors constantly pose imminent threats to our Philippine native chickens which include climate change, the influx of exotic breeds of chicken and the emergence of new avian diseases.

Cryopreservation allows the storage of cells that contain the genetic information of an organism for a longer period (Pegg, 2007; Barbas and Mascarenhas, 2009). The long shelf-life of frozen cells, like gametes, gives lengthy opportunities for improvements without losing their genetic resources. The use of this technology has been improved and widely adopted in mammalian species such as bulls (Ugur *et al*., 2019), rams (Ntemka *et al*., 2018), goats (Gangwar *et al*., 2016), and stallions (Alvarenga *et al*., 2016). However, semen from avian species is known to be more sensitive to cryopreservation than mammalian semen (Gould and Styperek, 1989). Avian sperm are long (80 to 90 μm) making them more susceptible to mechanical manipulations such as pipetting and centrifugation (Long, 2006), which are common methods used during the process of cryopreservation (Agca and Critser, 2002). Moreover, the avian sperm tail is approximately 8 times the length of the sperm head which also predisposes poultry sperm to be more sensitive to freezing injuries (Donoghue and Wishart, 2000). In fact, cryopreserved poultry sperm has a significantly lower fertility rate than any domestic mammalian species (Long, 2006). Although, many studies have been undertaken for several years to improve cryopreservation protocols in avian (Long *et al*., 2010; Ehling *et al*., 2012; Blanch *et al*., 2014; Madeddu *et al*., 2016; Telnoni *et al*., 2017; Khaeruddin *et al*., 2019; Thelie *et al*., 2019), these methods often harm spermatozoa biochemically and functionally, lowering their motility and causing morphological abnormalities (Long, 2006).

On the other hand, specimens that are cryopreserved may be used to extract genetic information. This makes cryopreservation an excellent tool for conserving our Philippine native chickens. Determining the proper cryopreservation technique can lead to several other benefits which include increased flock productivity as it may be used as a tool in aid of production through artificial insemination (AI). Moreover, it can also be used to control pathogen transmission (Ombelet and Robays, 2015), and as a technology for the adequate storage of diverse genetic resources (Pegg, 2007).

Recent studies have demonstrated that the commonly used cryoprotectant (CPA) for sperm cryopreservation is glycerol (Junaedi *et al*., 2016), but it has toxic and contraceptive effects on sperm (Holt, 2000). With this, dimethyl sulfoxide (DMSO) is seen as an alternative to glycerol due to its rapid penetration into the cell and lower molecular weight relative to glycerol. DMSO has also been used successfully to cryopreserve sperm in other species like goats (Kundu *et al*., 2000). In another study, the thawing of frozen avian sperm at a temperature of 37° C for 30 seconds has shown better post-thawing results (Miranda *et al*., 2018).

Overall, this study aimed to characterize the Paraoakan native chicken semen and compare their post-thawing semen characteristics after cryopreservation using either 8% glycerol or 10% DMSO, as CPA, in an AU-based extender. Moreover, this study sought to contribute some basic and applied information on Paraoakan native chicken for their propagation and conservation.

MATERIALS AND METHODS

This study was carried out from September 2020 to January 2021. All animals were maintained at the University Animal Farm (UAF) (14°09'24.4"N, 121°15'06.6"E) and underwent a flock health program instituted in the farm. Semen processing and other laboratory procedures were performed at the Animal Physiology Laboratory, Institute of Animal Science (IAS), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB).

Thirteen (13) sexually mature and healthy Paraoakan native roosters were housed in cages individually, labeled as Paraoakan1 to Paraoakan13, and attended by one caretaker. Experimental roosters were maintained in a stress-free environment under proper husbandry conditions on the farm. The roosters follow an optimized daily routine and were fed with standard commercial diets with water offered *ad libitum*. Semen samples were collected individually by abdominal massage and siphoned with a sterilized, 1cc syringe per animal. The samples were first put on a sealed and sterilized glass funnel before siphoning. Collected semen samples were immediately brought to the Animal Physiology Laboratory for evaluation and processing. All procedures were following the recommendations of the Institutional Animal Care and Use Committee (IACUC) with assigned protocol number CAFS-2018-006.

The semen samples collected were placed in a microcentrifuge tube per animal. The semen samples were diluted 1:45 with AU extender. About 1.5µL of the extended sample was pipetted to a glass slide and spread with a coverslip for Computer Assisted Sperm Analysis (CASA) and the motility of each sperm sample per animal was determined. The animals were ranked from lowest to highest motility after several semen collections and evaluations. Samples with similar total motility were grouped creating 4 batches: Poor1, Poor2, Good1, and Good2. The samples were pooled according to their batch designation.

Upon pooling of poor and good batches, the samples were diluted with AU extender to 1:1 ratio for cryopreservation and determination of sperm concentration. An aliquot (10% of total volume) of the pooled samples was diluted to 1:45 ratio using AU extender optimized for Computer Assisted Sperm Analysis (CASA) and viability testing. The samples were diluted using AU extender which is composed of 0.2g D-glucose, 0.4g D- fructose, 0.4g sugar, 0.45g sodium citrate, 0.42g monosodium glutamate (MSG), 0.2g glycine, 0.002g ethylenediaminetetraacetic acid (EDTA) and 100 mL distilled water.

The pooled semen samples were characterized prior to processing. The color and consistency of individual semen samples per batch were examined by visual appraisal. Total volume was determined using the 1cc syringe used for sample collection. Power of hydrogen or pH was determined using pH strips. Sperm count or sperm concentration was determined by manually counting the number of cells by Neubauer haemocytometry (Capitan and Palad, 1999). Briefly, the diluted semen sample was drawn up to the 0.5 mark of a dilution pipette and mixed with the staining solution until it reached the 101 or 11 mark on the dilution pipette. The pipette was gently swirled for thorough mixing, making sure that the sperm cells were evenly distributed. The mixture was placed on the counting chamber of a Neubauer slide with a cover slip for spreading. The slide was placed on the microscope stage and was observed under 400x magnification. The number of cells inside the 4 corner boxes and the middle box of the 5x5 counting chamber was determined. Viability was determined by eosin-nigrosin staining through phase contrast microscopy. The eosin-nigrosin stain used

was composed of 1.67 g eosin and 10 g nigrosin in 100mL sterile distilled water containing 2.9 g of sodium citrate (Capitan and Palad, 1999). The diluted pooled samples were mixed with the stain at a 1:1 ratio on a glass slide and smeared by sliding another glass slide on top. Prepared slides were immediately dried using a hotplate and viewed under 1000x total magnification. The total motility was determined using Computer Assisted Sperm Analysis – Animal Breeder Software. A glass slide with 1.5µL of diluted semen sample (1:45 ratio) spread with a cover slip was placed on the stage of the microscope equipped with a high-performance camera connected to the Animal Breeder Software. Different settings were set (i.e., storage location, animal species, animal ID) and the data were presented after capturing. An average of 5 frames was recorded to ensure the accuracy of data on the motility of each sample.

The cryopreservation protocol used in this experiment was modified based on the study of Miranda et. al (2018). Instead of using Kobidil extender, which is not locally available in the Philippines, AU extender (Gerzilov *et al*., 2011) was used. AU extender was prepared by mixing 0.40 g glucose, 0.80 g fructose, 0.80 g sucrose, 0.90 g sodium citrate, 0.84 g sodium glutamate, 0.40 g glycine, and 0.04 g EDTA with 100 ml sterile distilled water. Pooled semen samples from each batch were diluted 1:1 with AU extender and placed in a refrigerator (4°C to 9°C) for 45 mins. The cryoprotective medium was prepared by diluting 10% DMSO or 8% glycerol with AU. The amount of AU to dilute the CPAs was adjusted to obtain a final sperm count of 200,000,000 cells per 0.5 mL aliquot of sample. The prepared cryoprotective medium was added to each extended sample after 45 mins. Semen with cryoprotective medium was siphoned in 0.5 mL straws and sealed with straw powder. Samples were kept inside the refrigerator for 15 minutes before equilibrating them in liquid nitrogen vapor (5 cm above the liquid nitrogen surface) for another 15 mins. After equilibration, samples were stored in liquid nitrogen until thawing.

The cryopreserved samples were thawed at 37°C for 30 seconds according to Miranda *et al*. (2018). The post-thawing motility was determined using the same procedure used for the determination of motility before cryopreservation. The 1:45 dilution optimized for CASA was achieved by diluting the thawed samples with AU extender. The post-thawing viability was determined using the same procedure used for the determination of viability before cryopreservation.

Shapiro-Wilk Test was used to test for normality whereas Levene's Test was used to test for homoscedasticity. Since the data were not normally distributed and the variances were heterogenous, Wilcoxon Rank Sum Test was used to compare the post-thawing semen quality (% motile, % progressive motility, and % viability) of samples cryopreserved using 8% glycerol and 10% DMSO. Wilcoxon Rank Sum Test was also used to compare the Good and Poor batches. Lastly, Kendall Tau correlation was used to determine the relationship between motility and viability.

RESULTS AND DISCUSSION

Samples were collected from thirteen (13) Paraoakan roosters from the Institute of Animal Science – University Animal Farm, University of the Philippines Los Baños for this study. Three (3) of those were considered extraneous due to their initial batch designation (i.e. Paraoakan11, Paraoakan12 and Paraoakan13). For the ten (10) remaining Paraoakan roosters, semen samples were collected thrice a week for 4 weeks, which were then

evaluated in the Animal Physiology Laboratory. The quantitative and qualitative semen characteristics evaluated were consistency, pH, sperm count, initial sperm motility, initial sperm viability, and initial sperm progressive motility. The initial motility evaluated using CASA after several collections was the sole criterion in grouping the Paraoakan roosters. Those with similar initial percent total motility were grouped to create batches, particularly Good1, Good2, Poor1, and Poor2, summarized in Table 1.

Samples from only good and poor batches were pooled and obtained for further observation. Particularly, 10 collections were made from the good batch (Good1 and Good2) and 10 collections were made from the poor batch (Poor1 and Poor2) The initial motility of those pooled batches was again obtained. The range of reported normal rooster semen motility was between 40% to 80% (Lake *et al*., 1966; Omeje and Marire, 1990; King *et al*., 2000; Malik *et al*., 2013; Kesniel *et al*., 2016). Although semen motility of 30% to 40% is still acceptable, it remains undesirable according to the Food and Agriculture Organization (2012). The values observed in the current study were fluctuating; the poor batch has 16.90% as the lowest initial motility and 74.10% as the highest while the good batch has 53.70% as the lowest and 93.40% as the highest. The average initial sperm motility in both good and poor batches is 60.23 ± 18.16%. This value is close to the reported value of Kesniel *et al*. (2016) with a percent motility of 61.67 ± 7.64%. All observed semen characteristics prior to cryopreservation and thawing are summarized in Table 2.

The consistency varies from watery to thick creamy, with 39.6% identified as watery from the collected samples. The pH range of the samples from the good batch was 6.6 to 8.0, while that of the poor batch was 6.8 to 8.0. The mean pH for the good and poor batches were 7.09 and 7.11, respectively, with an overall mean pH of 7.11. The ejaculate volume varies from 0.04 to 0.48 regardless of the batch. On the contrary, higher semen volumes were reported by Tarif (2013) in Sasso roosters and Adeoye *et al*. (2018) in Nigerian local chickens. For the sperm count of the sample diluted to 1:1 ratio, the highest and lowest in the good batch were 1772 and 252, respectively, with a mean of 772.69, while that in the poor batch were 2953 and 226, respectively, with a mean of 1294.13.

Also shown in Table 2 are the initial viability of the semen samples. Viability data were obtained in terms of per 200 cells but are reported as percentage. The data showed higher consistency in values for both batches. This is apparent in the relatively close means of the good and poor batches, with values 88.45% and 68.50%, respectively. The poor batch has 45.50% as the lowest initial viability and 82.00% as the highest while the good batch has 77.50% as the lowest and 99.50% as the highest. According to the study of Baguio and Capitan (2009), diluted semen viability of *Gallus gallus domesticus* has an average of 73.3% near to the computed average semen viability for Paraoakan rooster with 78.48%.

Table 1. Batch designation of the thirteen (13) Paraoakan roosters.

Good1	Good2	Mid	Poor1	Poor2
Paraoakan4	Paraoakan3	Paraoakan1	Paraoakan6	Paraoakan7
Paraoakan10	Paraoakan5	Paraoakan2	Paraoakan8	Paraoakan11
Paraoakan13*	Paraoakan9	Paraoakan12		

*mortality (January 2021)

Table 2. Range values and means of semen characteristics.

Parameters		Parameter Range/ Value	Mean	SD
Initial Motility, %	Good	53.70 – 93.40	73.37	±10.2114
	Poor	16.90 – 74.10	47.08	±14.4515
	All	16.90 – 93.40	60.23	±18.1595
Consistency	All	Watery*	N/A	N/A
pH	Good	6.60 – 8.00	7.09	±0.2571
	Poor	6.80 – 8.00	7.11	±0.2124
	All	6.60 – 8.00	7.11	±0.2373
Sperm Count, $x10^9$	Good	2.52 – 17.72	7.73	±4.6721
	Poor	2.26 – 19.53	11.94	±5.4556
	All	2.26 – 19.53	9.83	±5.4368
Ejaculate Volume	Good	0.14 – 0.48	0.29	±0.1089
	Poor	0.04 – 0.34	0.18	±0.0931
	All	0.04 – 0.48	0.24	±0.1140
Initial Viability, %	Good	77.50 – 99.50	88.45	±6.7100
	Poor	45.50 – 82.00	68.50	±10.8821
	All	45.50 – 99.50	78.48	±13.4788
Initial Progressive Motility, %	Good	2.70 – 30.80	12.54	±6.1757
	Poor	0.90 – 13.10	5.98	±3.2029
	All	0.90 – 30.80	6.31	±6.9154

*39.6% watery, 34.4% thin creamy, 26.0% thick creamy

The sperm motility and progressive motility of pooled semen sample per batch was evaluated using CASA, while the viability was assessed using eosin- nigrosin staining or live-dead staining. This was done prior to cryopreservation and after thawing. In determining the difference between the two CPAs (DMSO and glycerol), the post-thawing variables are set to be the following: %Motility, %Progressive Motility, and %Viability. Range and mean values of the said post-thawing semen parameters are summarized in Table 3. Meanwhile, the response of the samples to cryopreservation with either the two CPAs using pooled semen samples from the two batches (good and poor) are also identified along with the correlation of %Motility and %Viability as shown in Table 4.

Cryopreservation has been reported to cause changes in sperm qualities (Wooley and Richardson, 1978). Hence, the proportion of fully functional sperm that retain after freeze-thaw is low (Holt, 1997). To compare semen quality after cryopreservation between the two cryoprotectants (DMSO vs. glycerol) across the two batches (good and poor), three parameters were taken particularly %Motility, %Progressive Motility, and %Viability. This was done using Wilcoxon Rank Sum Test since Shapiro-Wilk Test for Normality has been violated by the three said parameters. Along with this, only the data for %Progressive Motility follow the homoscedasticity assumption of the Pearson correlation but violating the

Table 3. Post-thawing range values and means of semen characteristics.

Parameters	Treatment	Batch	Parameter Range/ Value	Mean	SD
Post-thawing Motility, %	Glycerol	Good	2.3 – 49.6	18.45	±13.7668
		Poor	7.7 – 44.3	20.00	±10.5201
	DMSO	Good	6.3 – 34.8	15.36	±7.5873
		Poor	2.4 – 25.6	12.98	±5.2899
Post-thawing Progressive Motility, %	Glycerol	Good	0.3 – 7.4	1.89	±1.8879
		Poor	0.7 – 3.8	1.88	±0.9924
	DMSO	Good	0.2 – 4.6	1.77	±1.0017
		Poor	0 – 2.5	1.24	±0.8133
Post-thawing Viability, %	Glycerol	Good	9.0 – 40.0	16.53	±8.0418
		Poor	10.0 – 40.0	20.78	±8.6638
	DMSO	Good	4.5 – 37.5	14.98	±7.0701
		Poor	7.5 – 21.5	13.51	±3.9444

Table 4. Wilcoxon Rank Sum Test for Glycerol vs. DMSO.

Trait	W	*P*-value	Conclusion
% Motility	606.5000	0.0316	Glycerol > DMSO
% Progressive Motility	712.5000	0.4018	No significant difference
% Viability	533.5000	0.0052	Glycerol > DMSO

assumption for normality makes Wilcoxon Rank Sum Test more fit to use for these variables. Generally, at 5% level of significance ($P<0.05$), DMSO has shown a significantly lower median %Motile and %Viability than glycerol whereas there appears to be no significant difference in the median %Progressive Motility between the two CPAs.

To compare the overall response between batch samples (good vs. poor) to cryopreservation (i.e., across CPAs), the %Decrease in all three parameters is taken into consideration. This was done using Wilcoxon Rank Sum Test since Shapiro-Wilk Test for Normality has been violated by the three said parameters. Based on Table 5, the median decrease for the good batch (Good1 and Good2) in the three parameters (%Motile, %Progressive Motility and %Viability) are greater than that of the poor batches (Poor1 and Poor2) at 5% level of significance ($P<0.05$). Several factors may be the reason for significant differences between these two batches, hence, further research regarding this would be an interesting subject.

To determine if motility is correlated with viability, Table 6 displays a strong positive monotonous relationship between motility and viability of the samples collected using Kendall correlation due to violation of both normality and homoscedasticity assumptions. The results are similar to the outcome of the study of Spaleková *et al*. (2013) on bull spermatozoa where it was concluded that there is a close relationship between motility and viability parameters of frozen-thawed bull spermatozoa. This proves that sperm motility and

Table 5. Wilcoxon Rank Sum Test for Good batch vs. Poor batch.

Trait	W	*P*-value	Conclusion
% Decrease in Motility	399.5000	0.0001	Good > Poor
% Decrease in Progressive Motility	383.0000	0.000	Good > Poor
% Decrease in Viability	421.0000	0.0001	Good > Poor

Table 6. Kendall Tau correlation coefficient for motility and viability.

	Coefficients		*P*-value	
	Motility	Viability	Motility	Viability
Motility	1.0000	0.6088	0.0000	0.0000
Viability	0.6088	1.0000	0.0000	0.0000

viability are two parameters that are strongly correlated since most spermatozoa presenting any type of motility are viable.

Albeit glycerol has been routinely used as a cryoprotectant in the cryopreservation of male germplasm in many avian and mammalian species, studies are still being conducted to seek alternatives (Rakha *et al*., 2018a; Rakha *et al*., 2018b) due to its contraceptive effects (Holt, 2000). The data on the semen quality after cryopreservation comparing the two CPAs on motility, progressive motility, and viability of Paraoakan rooster semen indicates that glycerol is still more suitable for semen cryopreservation in chicken as compared to DMSO. The results were consistent with the findings from previous studies (Farshad *et al*., 2009; Junaedi, 2016; Svoradová *et al*., 2018). It is also reported that the decrease in motility, progressive motility and viability is greater in good batch than in poor batch.

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