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Suitability of Sugarcane Extract as a Local Extender and the Use of Either DMSO or Glycerol as Cryoprotectant for the Cryopreservation of the Banaba Native Breed Chicken (*Gallus gallus domesticus*) Semen

Abdul-Rahaman S. Salifu[1,3]*, Jose A.N. Bautista[1], Antonio A. Rayos[1], Josefina T. Dizon[2], and Percival P. Sangel[1]

[1]Institute of Animal Science, College of Agriculture and Food Science, University of the Philippines Los Baños, College 4031 Laguna, Philippines
[2]Institute for Governance and Rural Development, College of Public Affairs and Development, University of the Philippines Los Baños, College, Laguna, Philippines
[3]Department of Ecological Agriculture, School of Applied Science and Arts, Bolgatanga Technical University, Box 767, Bolgatanga, Ghana

The experiment was designed to test the suitability of sugarcane extract (SE) as a local extender for the cryopreservation of the Banaba native chicken semen by comparing the effects of different cryoprotective agent concentrations [*i.e.* glycerol and dimethyl sulfoxide (DMSO)] and thawing temperatures on the post-thaw semen quality. Twelve (12) 29-month-old Banaba native roosters served as semen donors. Only samples that passed the preliminary semen quality assessment of > 70% motility and morphology were further processed and analyzed using a computer-assisted semen analyzer (CASA). Pooled ejaculate samples were cryopreserved with SE extender containing 3, 5, 7, or 9% (T3%, T5%, T7%, and T9%) glycerol or DMSO concentration levels. The results showed that the inclusion level of cryoprotectants and thawing temperatures have no significant effect on the percent motile sperm, percent progressive sperm, percent normal morphology, and percent viability. The type of cryoprotectant used in cryopreservation of the semen had a significant effect on all the semen quality parameters. Semen cryopreserved with glycerol had significantly higher values compared to DMSO in all semen quality variables.

Keywords: Banaba native chicken, cryopreservation, dimethyl sulfoxide, glycerol, sugarcane extract

INTRODUCTION

Natural extenders are alternatives to chemical extenders in semen cryopreservation of various farm animals and non-domestic species. Natural semen extenders are cheaper, non-toxic, and effective in preserving animal sperm (Silva *et al*. 2012). These natural extenders have been studied in goats (Daramola *et al*. 2016), dogs (Cardoso *et al*. 2003; Šichtař *et al*. 2016), cattle (El-Sheshtawy *et al*. 2017), pigs (Castro *et al*. 2020; Akandi *et al*. 2015), horses (El-Sheshtawy *et al*. 2016), fish (Onyia *et al*. 2017; Muchlisin *et al*. 2010; Muchlisin and Nandiah 2015), collared peccaries (Silva *et al*. 2012), and chickens (Esguerra *et al*. 2020; Rochmi and Sofyan 2019; Malik *et al*. 2019). The cryoprotective properties of natural extenders are associated with their strong antioxidant ability, protecting spermatozoa during the cryopreservation process from oxidative damage (El-Sheshtawy *et al*. 2017; Daramola *et al*. 2016).

*Corresponding author: assalifu@up.edu.ph
rana1991@gmail.com

Sugarcane extract (SE) as a natural product contains appreciable amounts of total sugars, minerals, vitamins, antimicrobial and cytoprotective effects, and antioxidants (Chinnadurai 2017; Islam 2017; Akandi *et al*. 2015; Abbas *et al*. 2013) for the protection of sperm viability and motility. The suitability of SE-based extenders for processing semen has been demonstrated in fish by Muchlisin *et al*. (2010) and Onyia *et al*. (2017).

Glycerol and dimethyl sulfoxide (DMSO) are penetrating cryoprotectants generally used for the cryopreservation of mammalian spermatozoa (Silva *et al*. 2012). Despite the contraceptive effect of glycerol and the toxic effect of DMSO, they are still in use for sperm cryopreservation. The optimum cryoprotectant concentration is the art of balancing its toxic and protective effects, as higher concentration levels decrease sperm fertilizing ability (Silva *et al*. 2012). Moreover, egg yolk is normally added to semen extenders for its protective and buffering effects on the sperm during thermal shock (Anand *et al*. 2014).

The main objective of this study was to determine the efficacy of SE-based extender with different concentrations of glycerol and DMSO (3, 5, 7, or 9%) as an alternative freezing medium for cryopreservation of Banaba native chicken spermatozoa.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee of the University of the Philippines Los Baños (UPLB) approved the animal care and sample collection procedures with the allotted protocol number CAFS-2018-006.

Semen Collection and Initial Evaluation

Semen was collected from 12 sexually mature Banaba native roosters (not proven breeders) aged 29 months old with an average body weight of 2.4 kg, reared at the University Animal Farm in *Barangay* Putho-Tuntungin, Los Baños, Laguna, Philippines with location coordinates 14°09'24.4"N, 121°15'06.6"E, using the abdominal massage method, twice per week for 7 weeks at 07:00 AM every collection day. Semen collection usually takes about 10 min for the entire collection period. The semen samples in Indoplas® sterile disposable 1-mL syringes were placed inside a properly disinfected foam-padded ice box with sterile cloth and transported to the laboratory within 10–15 min for processing and evaluation. The evaluated variables were sperm cell concentration, percent motile sperm, percent sperm morphology, and percent viability. Only ejaculates with more than 70% motility and morphologically normal spermatozoa were diluted and used for further processing using computer-assisted semen analyzer (CASA) (Ceros II, IMV Technologies, China). The ejaculates were pooled to eliminate individual variability effects among the donor roosters. The semen was given 10-min holding time at room temperature before dilution.

Semen Processing and Freezing

The pooled semen was diluted in the ratio of 1 part of semen to 2 parts of sugarcane-based extender (semen: extender) and then mixed gently. The diluted semen was allowed to stand at room temperature for 10–15 min and was divided into eight equal parts – four each for glycerol and DMSO cryoprotectants. All semen samples in 15-mL conical tubes with appropriate labels were equilibrated for 1 h at 3–5 °C in a refrigerator. After cooling, additional SE extender with either glycerol or DMSO final inclusion rates of 3, 5, 7, and 9% (T3%, T5%, T7%, and T9%) (also at 3–5 °C) were slowly added to their respective semen samples with a final sperm concentration of ~ 400 million cells/mL. The inclusion rates of the cryoprotectants were based on the available literature (Gill *et al*. 1996; Sontakke *et al.* 2004; Roushdy *et al*. 2014; Rakha *et al*. 2016; Telnoni *et al*. 2017). Aliquot 0.5 mL of semen with a freezing extender was aspirated into 1.5-mL labeled microcentrifuge tubes (Eppendorf tubes) and caps were secured with parafilm. Each treatment was labeled using paper tape and a permanent marker on the sides of the microcentrifuge tubes. The semen in the microcentrifuge tubes was arranged in a freezing rack and placed again in the refrigerator at 3–5 °C for another 1 h. At the end of the equilibration period, the tubes with the semen in the freezing rack were exposed to liquid nitrogen vapor at 10 cm above the liquid nitrogen in a nitrogen tank for 15 min. The tubes were then plunged into liquid nitrogen for storage at –196 °C for 3 d. Each collection batch serves as a replicate.

Semen Quality Assessment

Each treatment of the frozen semen was divided into two groups and thawed at two different temperatures: 5 and 37 °C. The frozen samples thawed at 5 °C were kept in a refrigerator for 25–30 min, whereas those thawed at 37 °C were put in a water bath for 1 min. Sperm motility and morphology were evaluated using the Gallus setup/ module of CASA (Ceros II, IMV Technologies, China) with a frame capture speed of 60 Hz and camera exposure of 4 ms. Five (5) frames were captured for every analysis.

Sperm viability was determined using the eosin-nigrosin method, as described by Capitan and Palad (1999). The stain was heated to a temperature of 37 °C, and two drops of the stain were placed on a fresh and warm glass slide. Then, a drop of semen was added to the slide, mixed, and spread a little on the slide. A second glass slide was

placed over the smear, and the excess stain was removed by wiping the edges of the slides with paper towels. After wiping the edges, the two slides were drawn apart and rapidly dried in a slide dryer (Fisher Scientific, USA) at 37 °C for 30 min. The dried slides were then examined under the oil immersion objective of the microscope. Partially stained sperm cells were counted as dead. A total of 333 sperms were counted, and the percentage of live sperms was computed by the formula:

$$\textit{Percentage of live sperm} = \frac{\textit{Number of live sperm counted}}{\textit{Number of sperm counted (live and dead)}} \times 100 \quad (1)$$

Preparation and Composition of the Extender Used in the Experiment

The 2004-1011 sugarcane variety obtained from the UPLB Institute of Plant Breeding was used for the experiment. The freshly cut sugarcane pieces with a maximum of four internodes were washed clean under running water and kept in a refrigerator for use. The maximum storage duration of each batch of sugarcane was 4 days. To prepare the SE extender, the skin of the sugarcane of the one-piece internode was peeled off and rinsed under running water. After peeling and washing the sugarcane, two cuts were made along the cylindrical shape dividing it into four quarters. It was then placed in the juice extractor and pressed to express the liquid. The liquid was then filtered using sperm filtering paper together with cheesecloth, and the extract was used as a component of the extender. The optimized composition of the SE extender used in the experiment was obtained through the "trial and error method" of different SE concentrations before arriving at the 40% inclusion rate, as shown in Table 1.

Table 1. Optimized composition of sugarcane extract extender for Banaba native chicken.

Component	Composition (mL)
Sugarcane extract	40
Distilled water	30
Egg yolk-citrate extender	30
pH	6.79
Total	100

Statistical Analyses

All data gathered were first tested for normality and homoscedasticity using Shapiro-Wilk's test and Levene's test, respectively. After satisfying both assumptions, the data on fresh diluted semen parameters (*i.e.* % total motility, % progressive motility, % normal morphology, and % viability) were analyzed using one-way ANOVA. Meanwhile, two-way ANOVA was used for the data analysis of frozen-thawed semen parameters (*i.e.* % total motility, % progressive motility, % normal morphology, and % viability) with treatment (inclusion levels of cryoprotectant) and thawing temperature as main effects. One-way ANOVA was also used to compare the data on frozen-thawed semen parameters between cryoprotectants (*i.e.* glycerol vs DMSO) or thawing temperatures (*i.e.* 5 °C *vs.* 37 °C). Tukey's LSD was used as a *post hoc* analysis tool to determine the level of significance among the means at 5%. All statistical analyses were done using STATA V 15 (StataCorp LLC, USA).

RESULTS AND DISCUSSION

Characteristics of Fresh Semen of Banaba Native Chicken

The fresh semen quality characteristics of Banaba native chicken are summarized in Table 2. Prior to glycerol and DMSO additions as treatments, semen samples diluted with SE were analyzed for total motility, progressive motility, morphology, and viability. There were no significant ($p > 0.05$) differences among the fresh semen quality parameters assessed in the samples of the Banaba native chicken assigned to the different treatment groups (T1–T4). The percent total motility values for the various treatments were 82.98 ± 3.45, 78.14 ± 2.31, 78.14 ± 2.31, and 79.24 ± 2.92% for the T1 (3%), T2 (5%), T3 (7%), and T4 (9%), respectively.

The total motility values in this current study are higher than those of Junaedi *et al*. (2017) at 76.66 ± 1.66% and Gill *et al*. (1996) at 66.9 ± 5.2% but similar to Telnoni *et al*. (2017) at 82.75 ± 1.38%.

However, Khaeruddin *et al*. (2019) reported a higher value of 90.00 ± 1.75% for Kampung roosters. Tabatabaei *et al*. (2010) reported mean sperm motility values of 78 and 86% for the Ross and indigenous breeds, respectively, in a semen quality comparative study of these breeds. Baguio and Capitan (2008) observed 81.7% sperm motility in fresh undiluted semen of the Philippine native chicken and between 51.2 and 71.2% in diluted semen. This variation may be attributed to breed differences between roosters and the type of extender used in the experiment. The percent progressive motility was similar among treatments and showed progressive motility ranged from 39.22 ± 3.88 to 44.52 ± 2.79%. This was obviously good because it was half the value of the total motility observed.

The success of a breeding program hinges on the morphologically normal sperm cells as it correlates positively with fertility. The normal sperm morphology

Table 2. Mean (± SEM) fresh semen characteristics of Banaba native chicken diluted with sugarcane extract extender (n = 5).

Treatment	Parameter			
	TM (%)	PM (%)	Morphology (%)	Viability (%)
T1 (3%)	82.98 ± 3.45	44.52 ± 2.79	89.92 ± 4.023	94.20 ± 0.74
T2 (5%)	78.14 ± 2.31	39.48 ± 4.06	91.06 ± 1.84	94.30 ± 0.73
T3 (7%)	78.14 ± 2.31	39.48 ± 4.06	91.06 ± 1.840	94.30 ± 0.73
T4 (9%)	79.24 ± 2.92	39.22 ± 3.88	91.80 ± 4.81	94.10 ± 0.74
***p*-value**	0.578	0.706	0.495	0.997

[TM] total motility; [PM] progressive motility

reported herein was between 84.92 ± 4.02 and 91.06 ± 1.84%, as shown in Table 2. The reported values in the present study are higher than those recorded values of 75–79% by Guieb *et al*. (2016) on the Philippine native chicken using different extenders. In contrast, Rochmi and Sofyan (2019) reported 93.5% of normal sperm morphology in fresh rooster semen. This means that the sperm morphology in this present study was above 80% and within acceptable limits.

Sperm viability is the percentage of live/dead spermatozoa. The sperm viability of the fresh Banaba native chicken ranges from 94.10 ± 0.74 to 94.30 ± 0.73%. The results in this report are greater than the reported sperm viability of 90.78 ± 1.59% by Telnoni *et al*. (2017) in Sentul Kampong Kedu (SK Kedu) chicken but lower than the recorded value of 97.07 ± 0.42% in Kampung roosters by Khaeruddin *et al*. (2019). Baguio and Capitan (2008) reported sperm viability of 85.40% in undiluted semen and 54.6-73.3% in diluted semen of the Philippine native roosters. Similarly, Rochmi and Sofyan (2019) reported a 93±0.50% sperm viability for fresh rooster spermatozoa, whereas Masoudi *et al*. (2019) reported sperm viability values of 91.20 ± 1.90 to 94.30 ± 1.90% in a diluted sperm. The results in this report suggest that the processing procedures (*i.e.* chicken selection, semen collection, semen handling during transportation, dilution rate, *etc.*) used prior to freezing were able to maintain good quality semen samples of Banaba native chicken which is required for cryopreservation.

Effects of Different DMSO Inclusion Levels and Thawing Temperatures on the Frozen-thawed Sperm Characteristics of Banaba Native Chicken

As presented in Table 3, the use of DMSO at different inclusion levels at different thawing temperatures did not affect the percent sperm total motility, percent progressive motility, percent normal morphology, and percent viability of Banaba native chicken.

The results also showed no significant ($p > 0.05$) treatment x thawing temperature interaction effect and treatment or thawing temperatures' main effect on the observed semen characteristics. However, percent total motility, percent progressive motility, and percent morphology after thawing at 5 °C increased with increasing DMSO concentration up to 7% with a slight decrease in observed values at 9% DMSO. Meanwhile, the same observed semen parameters decreased with increasing DMSO concentration of samples thawed at 37 °C.

Viability (%), on the other hand, showed a different trend with increasing DMSO concentration (T1 > T2 < T3 < T4) in both thawing temperatures. Similar findings were reported by Telnoni *et al*. (2017) in Sentul Kampong Kedu (SK Kedu) chicken where DMSO concentrations did not show any significant effect on sperm motility and viability in Beltsville poultry semen extender. Cardoso *et al*. (2020), reported no significant effect of thawing temperatures (37 °C/ 30 s *vs.* 5 °C/ 1 min) and DMSO concentrations (4% *vs.* 8%) on spermatozoa quality. Junaedi *et al*. (2017) recorded increased % motility and viability with increasing dimethylacetamide (DMA) concentration after thawing Kampong broiler semen at 37 °C/ 30 s using Lactated Ringer's egg yolk as a based extender. In blue rock pigeons, Sontakke *et al.* (2004) reported a higher value of 39.2 ± 4.3 (DMSO 8%) of % motile spermatozoa compared to 8.6 ± 1.5 (DMSO 4%), clearly showing increasing concentration with increasing cryoprotection. Sikarwar *et al*.'s (2015) results in goats are also similar to the results in this current study, which showed increased sperm quality parameters with increasing concentration of DMSO up to 6% with no apparent changes at 8%.

Effects of Different Glycerol Inclusion Levels and Thawing Temperatures on the Frozen-thawed Sperm Characteristics of Banaba Native Chicken

As indicated in Table 4, with the exception of thawing temperature, treatment main effect, and treatment x thawing temperature interaction effect had no influence on the percent sperm total motility, percent progressive motility, percent normal morphology, and percent viability of Banaba native chickens.

Table 3. Mean (± SEM) semen characteristics of Banaba native chicken cryopreserved using DMSO at different inclusion levels and thawed at two different temperatures (n = 5).

Treatment	Thawing temp.	Parameter			
		TM (%)	PM (%)	Morphology (%)	Viability (%)
T1 (3%)	5°C	37.03 ± 3.46	6.70 ± 1.61	67.00 ± 7.57	57.83 ± 5.17
T2 (5%)	5°C	39.50 ± 3.31	7.00 ± 1.37	83.00 ± 4.75	55.17 ± 5.34
T3 (7%)	5°C	43.70 ± 6.99	7.73 ± 1.52	79.90 ± 2.05	58.67 ± 5.24
T4 (9%)	5°C	38.23 ± 6.74	5.60 ± 1.45	74.03 ± 6.12	62.83 ± 10.48
T1 (3%)	37°C	49.97 ± 4.20	9.23 ± 0.24	81.67 ± 6.86	62.17 ± 5.13
T2 (5%)	37°C	38.50 ± 5.36	6.13 ± 2.23	80.90 ± 6.11	50.50 ± 5.92
T3 (7%)	37°C	38.87 ±3 .45	4.83 ± 0.99	73.10 ± 3.25	57.17 ± 6.10
T4 (9%)	37°C	37.70 ± 3.92	4.56 ± 0.27	62.83 ± 6.43	62.33 ± 4.69
			p-values		
Treatment		0.796	0.254	0.164	0.476
Thawing temperature		0.703	0.567	0.740	0.897
Treatment x thawing temp.		0.488	0.289	0.157	0.910

[TM] total motility; [PM] progressive motility; [Temp] temperature.

Table 4. Mean (± SEM) semen characteristics of Banaba native chicken cryopreserved using glycerol at different levels and thawed at two different temperatures (n = 5).

Treatment	Thawing temp.	Parameter			
		TM (%)	PM (%)	Morphology (%)	Viability (%)
T1 (3%)	5°C	61.64 ± 3.56^{ab}	17.36 ± 4.25	87.90 ± 4.12	67.40 ± 4.72
T2 (5%)	5°C	64.98 ± 2.04^{ab}	13.20 ± 2.53	86.12 ± 4.65	68.50 ± 5.27
T3 (7%)	5°C	62.98 ± 4.32^{ab}	13.14 ± 2.85	90.68 ± 3.11	74.20 ± 4.53
T4 (9%)	5°C	68.68 ± 1.89^{a}	15.54 ± 2.18	86.70 ± 6.58	73.30 ± 4.49
T1 (3%)	37°C	60.68 ± 3.82^{ab}	12.08 ± 2.71	88.88 ± 5.48	64.80 ± 4.08
T2 (5%)	37°C	57.82 ± 4.08^{b}	13.84 ± 4.80	83.96 ± 6.73	65.10 ± 6.61
T3 (7%)	37°C	59.86 ± 2.52^{ab}	11.40 ± 2.72	91.30 ± 2.39	64.80 ± 3.10
T4 (9%)	37°C	59.24 ± 4.85^{ab}	16.64 ± 3.06	79.0 ± 6.17	74.20 ± 5.98
			p-values		
Treatment		0.840	0.623	0.414	0.429
Thawing Temperature		0.047	0.571	0.573	0.316
Treatment x thawing temp.		0.628	0.753	0.821	0.781

[$^{a, b}$] Means in the same column with different superscript letters are significantly different at 5%. [TM] total motility; [PM] progressive motility; [Temp] temperature.

The results showed that, at 5 °C thawing temperature, the percent total motility generally increased with increasing glycerol concentration with T4 (9%) numerically higher than the other treatments. The percent progressive motility decreased with increasing levels of glycerol concentration. The T1 (3%) numerically had a higher (17.36 ± 4.25%) value than the other three treatments, with T3 (7%) being the least (13.14 ± 2.85%). The percent morphology and percent viability showed a trend of increased average values with increasing glycerol concentration up to 7% with no further observable changes at 9%. However, at 37 °C thawing temperature, percent total motility showed a decreasing increasing trend with increasing glycerol concentration. The T1 (3%) gave the highest average value with the least coming from T2 (5%). Moreover, percent progressive motility and percent viability generally showed a trend of increased values with increasing glycerol concentration except for T3 (7%), whereas percent morphology was highest in T3 (7%), with T4 (9%) being the lowest.

Thawing temperature did not significantly ($p > 0.05$) influence the percent progressive motility, percent morphology, and percent viability. However, a significant ($p = 0.047$) effect of thawing temperature was observed on sperm total motility (Table 4). At 5 °C thawing temperature, T4 (9%) was statistically ($p < 0.05$) higher compared to the other treatments, which were statistically similar. Thawing at 37 °C, T1(3%), T3 (7%), and T4 (9%) were statistically similar, whereas T2 (5%) was statistically lower at a 5% confidence level. Comparatively, thawing at a low temperature (5 °C) gave numerically higher values than thawing at a higher temperature (37 °C) (Table 4).

The results from this current study are similar to those of Gill *et al*. (1996), who reported insignificant differences in sperm motility between two glycerol concentrations in post-thaw semen of roosters, though higher concentration corresponded with higher sperm motility. In contrast, Rakha *et al*. (2016) reported significantly higher sperm variables with increasing glycerol concentrations in cryopreserved semen in red jungle fowl.

In other species, similar observations have been made where increasing glycerol concentration did not significantly affect sperm quality parameters. In collared peccaries, Silva *et al*. (2012) reported similar results with regard to sperm quality characteristics. Sontakke *et al*. (2004) also recorded increasing motility with increasing levels of glycerol in blue rock pigeon post-thaw semen. In goats, Sharma *et al*. (2020) reported that glycerol concentration had no effect on progressive motility and hypoosmotic swelling test (HOST) response. In contrast, Sikarwar *et al*. (2015) and Roushdy *et al*. (2014) reported glycerol concentration beyond 7% had no apparent benefits in post-thaw motility and viability of sperm cells in goats and roosters, respectively, whereas Buhr *et al*. (2001) also reported concentration up to 4% in goat semen with no benefit thereafter for progressive motility. However, sperm motility index and motility in post-thaw semen increased with increasing glycerol concentrations, which differs significantly in human semen (Hammitt *et al*. 1988). Significant results with increasing glycerol concentration have also been reported in cryopreserved semen of cats by Villaverde *et al*. (2013).

Also, improved semen quality characteristics with increasing concentration have been reported in other cryoprotectants. Junaedi *et al*. (2017) reported improvement in motility and viability with increasing DMA concentration in Kampong broiler chicken semen. According to Junaedi *et al*. (2017), 9% DMA gave significantly higher values in motility and viability compared to 5 and 7%, respectively. However, Roushdy *et al*. (2014) reported decreased post-thawed motility and viability with increasing DMA concentration in rooster semen.

Effects of the Type of Cryoprotectant on the Frozen-thawed Semen Characteristics of Banaba Native Chicken

The effects of the type of cryoprotectant (*i.e.* DMSO or glycerol) on the assessed semen quality parameters are shown in Table 5.

The results indicated that there were statistical ($p < 0.05$) differences among cryoprotectants for percent motile sperm, percent progressive sperm, percent sperm morphology, and sperm viability. The glycerol as a cryoprotectant showed significantly higher average values for all the observed semen quality parameters. The average percent motile sperm in the glycerol-treated samples was 61.99 ± 1.39% with a corresponding percent progressive sperm of 14.15 ± 0.92% compared with 40.44 ± 1.80% and 6.48 ± 1.19% for percent motile sperm and percent progressive sperm, respectively in the DMSO-treated samples.

The average morphologically normal sperm value was 86.82 ± 1.71% and the average viability was 69.04 ± 1.67% in the glycerol-treated semen in contrast with 75.30 ± 2.21% and 58.33 ± 2.16% for morphologically normal sperm and average viability, respectively in the DMSO-treated semen. Similar findings were reported by Sikarwar *et al*. (2015) and Mapeka *et al*. (2009), wherein semen cryopreserved with glycerol showed statistically higher sperm motility, progressive motility, and viability post-thaw than DMSO cryoprotectant. Based on their results, they stated that goat and Kolbroek boar sperm can better be cryopreserved using glycerol as a cryoprotectant, which is in line with the findings in this current study.

Table 5. Mean (± SEM) effects of the DMSO and glycerol on frozen-thawed sperm characteristics of banaba native chicken (n = 5).

Cryoprotectant	Parameter			
	TM (%)	PM (%)	Morphology (%)	Viability (%)
DMSO	40.44 ± 1.80^{b}	6.48 ± 1.19^{b}	75.30 ± 2.21^{b}	58.33 ± 2.16^{b}
Glycerol	61.99 ± 1.39^{a}	14.15 ± 0.92^{a}	86.82 ± 1.71^{a}	69.04 ± 1.67^{a}
***p*-value**	0.000	0.000	0.000	0.000

[a, b] Means in the same column with different superscript letters are significantly different at 5%. [TM] total motility; [PM] progressive motility.

Table 6. Mean (± SEM) effects of thawing temperature on frozen-thawed sperm characteristics of Banaba native chicken (n = 5).

Thawing temperature	Parameter			
	TM (%)	PM (%)	Morphology (%)	Viability (%)
5 °C	52.52 ± 1.58	10.83 ± 1.05	81.96 ± 1.95	64.93 ± 1.9
37 °C	49.90 ± 1.58	9.79 ± 1.05	80.16 ± 1.95	62.44 ± 1.90
***p*-value**	0.240	0.480	0.509	0.352

[TM] total motility; [PM] progressive motility

Donoghue and Wishart (2000) stated that glycerol is less toxic to sperm cells and most efficient cryoprotectant, whereas DMSO is a highly toxic and the least efficient cryoprotectant. The poor performance of the DMSO-treated semen may be a result of the toxic effect of the DMSO on the sperm cells. On the other hand, the better performance of the glycerol-treated spermatozoa may be because of a synergistic effect of the glycerol and the egg yolk in the extender (Silva *et al*. 2012).

Effects of Thawing Temperature on Frozen-thawed Sperm Characteristics of Banaba Native Chicken

The effect of thawing temperature on the sperm quality parameters of Banaba native breed chicken is presented in Table 6. The thawing temperature has no significant ($p > 0.05$) effect on the percent motile sperm, percent progressive sperm, percent normal morphology, and percent viability. However, thawing semen samples at 5 °C gave numerically higher values for all semen quality parameters: motility (52.52 ± 1.58), progressive motility (10.83 ± 1.05), morphology (81.96 ± 1.95), and viability (64.93 ± 1.90) as against 49.90 ± 1.58, 9.79 ± 1.05, 80.16 ± 1.95, and 62.44 ± 1.90 for motility, progressive motility, morphology, and viability, respectively for semen samples thawed at 37 °C of the Banaba native chicken semen.

Miranda *et al*. (2017) recorded a non-significant effect of thawing temperatures on sperm total motility and progressive motility in chickens using DMA and methyl acetamide as cryoprotectants. However, in the same study, the thawing temperature at 5 °C significantly influenced the cryoprotective effect of ethylene glycol and dimethylformamide, when compared to 37 °C. Similarly, Mphaphathi *et al*. (2012) stated that thawing rooster's spermatozoa at 5 °C (45.5%) resulted in a significantly higher motility rate compared to thawing at 25 ˚C (17.6%) and 41 ˚C (0.7%) but observed no significant effect on sperm viability. This means that thawing rooster's semen at higher temperatures had a harmful effect on the sperm cells.

In contrast, the findings in this current report are in direct conflict with those of Iaffaldano *et al*. (2016) that thawing temperatures of 50 °C/ 10 s for sperm motility was significantly higher than 4 °C/ 5 min for turkey spermatozoa. Han *et al*. (2005) reported a significantly higher post-thaw sperm motility rate (62%) of duck spermatozoa thawed at 40 ˚C compared to 29% thawed at 20 ˚C. Thawing temperature seems to affect spermatozoa quality parameters in a species-specific manner (Cardoso *et al*. 2020).

CONCLUSION

The results of this study suggest that the 40% inclusion rate of the SE in the extender is suitable for the cryopreservation of Banaba native chicken semen. The type of cryoprotectant used in cryopreservation of the semen had a significant effect on all the semen quality parameters. Semen cryopreserved with glycerol had significantly higher values compared to DMSO in all semen quality variables. It can, therefore, be concluded that SE with glycerol as a cryoprotectant is able to protect the sperm cells of roosters during cryopreservation.

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STATEMENT ON CONFLICT OF INTEREST

All authors have no conflict of interest to declare.

REFERENCES

ABBAS SR, AHMAD SD, SABIR SM, SHAH AH, AWAN S, GOHAR M, ... , RAO AZ. 2013. Antioxidant activity, repair, and tolerance of oxidative DNA damage in different cultivars of sugarcane (*Saccharum officinarum*) leaves. Australian Journal of Crop Science 7(1): 40–45.

AKANDI A, UGWU SO, MACHEBE NS. 2015. Survivability of boar sperm stored under room temperature in extenders containing some natural products. Open Access Animal Physiology 7: 57–64.

ANAND M, YADAV S, SHUKLA P. 2014. Cryoprotectant in semen extender: from egg yolk to low-density lipoprotein (LDL). Livestock Research International 2(3): 48–53.

BAGUIO SS, CAPITAN SS. 2008. Motility, livability, and fertility of cock spermatozoa as influenced by day of collection, dilution, and cryopreservation. Philippine Journal of Veterinary Medicine 45: 109–117.

BUHR MM, FISER P, BAILEY JL, CURTIS EF. 2001. Cryopreservation in different concentrations of glycerol alters boar sperm and their membranes. Journal of Andrology 22(6): 961–969.

CAPITAN SS, PALAD OA. 1999. Artificial breeding of farm animals. University of the Philippines Los Baños, Laguna, Philippines.

CARDOSO B, SÁNCHEZ-AJOFRÍN I, CASTAÑO C, GARCÍA-ÁLVAREZ O, ESTESO MC, MAROTO-MORALES A, INIESTA-CUERDA M, GARDE JJ, SANTIAGO-MORENO J, SOLER AJ. 2020. Optimization of Sperm Cryopreservation Protocol for Peregrine Falcon (*Falco peregrinus*). Animals 10(4): 691. https://doi.org/10.3390/ani10040691

CARDOSO RDCS, SILVA AR, UCHOA DC, DA SILVA LDM. 2003. Cryopreservation of canine semen using a coconut water extender with egg yolk and three different glycerol concentrations. Theriogenology 59(3–4): 743–751.

CASTRO CH, DICHOSO GA, LANDICHO MM, SANGEL PP. 2020. Honey or pineapple juice as extender components for Quezon native and Duroc boar semen at different storage temperatures. Philippine Agricultural Scientist 103(4): 322–336.

CHINNADURAI C. 2017. Potential health benefits of sugarcane. In: Sugarcane Biotechnology: Challenges and Prospects. Cham: Springer. p. 1–12.

DARAMOLA JO, ADEKUNLE EO, OKE OE, ONAGBESAN OM, OYEWUSI IK, OYEWUSI JA. 2018. Effects of coconut (*Cocos nucifera*) water with or without egg-yolk on viability of cryopreserved buck spermatozoa. Animal Reproduction 13(2): 57–62.

DONOGHUE AM, WISHART GJ. 2000. Storage of poultry semen. Animal Reproduction Science 62(1–3): 213–232.

EL-SHESHTAWY RI, EL-BADRY DA, GAMAL A, EL-NATTAT W. S, ALMAATY AMA. 2016. Natural honey as a cryoprotectant to improve Arab stallion post-thawing sperm parameters. Asian Pacific Journal of Reproduction 5(4): 331–334.

EL-SHESHTAWY RI, EL-NATTAT WS, ALI GAD. 2017. Cryopreservation of cattle semen using coconut water extender with different glycerol concentrations. Asian Pacific Journal of Reproduction 6(6): 279–282.

ESGUERRA JPM, QUIMIO JMUPH, DICHOSO GA, JUNSAY CAL, MAGPANTAY VA, SANGEL PP. 2020. Coconut water with either tomato juice or garlic extract as extender components for Paraoakan native chicken semen at different storage temperatures. Philippine Journal of Science 149(1): 121–131.

GILL SPS, BUSS EG, MALLIS RJ. 1996. Cryopreservation of rooster semen in thirteen and sixteen percent glycerol. Poultry Science 75(2): 254–256.

GUIEB HKD, VALDEZ MB, AQUINO FP. 2016. Comparison of the quality of extended Philippine native chicken (*Gallus gallus domesticus*) sperm collected by abdominal massage method. American Journal of Biological and Pharmaceutical Research 3(1): 24–27.

HAMMITT DG, WALKER DL, WILLIAMSON RA. 1988. Concentration of glycerol required for optimal survival and *in vitro* fertilizing capacity of frozen sperm is dependent on cryopreservation medium. Fertility and Sterility 49(4): 680–687.

HAN XF, NIU ZY, LIU FZ, YANG CS. 2005. Effects of diluents, cryoprotectants, equilibration time, and thawing temperature on cryopreservation of duck semen. Int. J. Poult. Sci 4(4): 197–201.

IAFFALDANO N, DI IORIO M, MIRANDA M, ZANIBONI L, MANCHISI A, CEROLINI S. 2016. Cryopreserving turkey semen in straws and nitrogen vapour using DMSO or DMA: Effects of cryoprotectant concentration, freezing rate, and thawing rate on post-thaw semen quality. British Poultry Science 57(2): 264–270.

ISLAM MT. 2017. Sugarcane: Safety Concerns on its Cytogenotoxic Damage, While Hopes as a Source of Anticancer Agents. Research Journal of Food & Nutrition 1(1): 10–12.

JUNAEDI J, ARIFIANTINI RI, SUMANTRI C. 2017. The quality of Kampung broiler (KB) chicken frozen semen with DMA concentrations on yolk lactate ringer diluent. Chalaza Journal of Animal Husbandry 2(2): 19–24.

KHAERUDDIN K, KURNIAWAN ME, SOMAN S. 2019. Cryopreservation of kampung rooster semen using egg yolk diluent from four types of poultry with different concentrations. Jurnal Kedokteran Hewan-Indonesian Journal of Veterinary Sciences 13(3): 60–65.

MALIK A, INDAH A, ZAKIR MI, SAKIMAN NS. 2019. Cryopreservative effect of adding a honey solution to native chicken spermatozoa. Adv Anim Vet Sci 7(4): 261–264.

MAPEKA MH, LEHLOENYA KC, NEDAMBALE TL, SUTHERLAND B. 2009. Effect of cryoprotectant on the cryopreservation of South African Kolbroek pig semen. South African Journal of Animal Science 39(Supplement 1): 198–201.

MASOUDI R, SHARAFI M, POURAZADI L. 2019. Improvement of rooster semen quality using coenzyme Q10 during cooling storage in the Lake extender. Cryobiology 88: 87–91.

MIRANDA M, KULÍKOVÁ B, VAŠÍČEK J, OLEXIKOVA L, IAFFALDANO N, CHRENEK P. 2017. Effect of cryoprotectants and thawing temperatures on chicken sperm quality. Reproduction in Domestic Animals 53(1): 93–100.

MPHAPHATHI ML, LUSEBA D, SUTHERLAND B, NEDAMBALE TL. 2012. Comparison of slow freezing and vitrification methods for Venda cockerel's spermatozoa. Open Journal of Animal Sciences 2: 204–210.

MUCHLISIN ZA, NADIYA N, NADIAH WN, MUSMAN M, SITI-AZIZAH MN. 2010. Preliminary study on the natural extenders for artificial breeding of African catfish *Clarias gariepinus* (Burchell, 1822). AACL Bioflux 3(2): 119–124.

MUCHLISIN ZA, NANDIAH WN. 2015. Exploration of natural cryoprotectants for cryopreservation of African catfish, *Clarias gariepinus*, Burchell 1822 (Pisces: Clariidae) spermatozoa. Czech Journal of Animal Sciences 60(1): 10–15.

ONYIA LU, OCHOKWU IJ, MOHAMMED U. 2017. Effects of different sperm extenders on the fertility, hatchability, and survival of *Clarobranchus*. CARD International Journal of Medical Science and Applied Biosciences 2(3): 19–29.

RAKHA BA, ANSARI MS, HUSSAIN I, ANWAR M, AKHTER S, BLESBOIS E. 2016. Comparison of extenders for liquid storage of Indian Red Jungle Fowl (*Gallus gallus murghi*) spermatozoa. Avian Biology Research 9(3): 207–212.

ROCHMI SE, SOFYAN MS. 2019. A diluent containing coconut water, fructose, and chicken egg yolk increases rooster sperm quality at 5 C. Veterinary World 12(7): 1116–1120.

ROUSHDY K, EL-SHERBIENY MA, EL-GANY F, EL-SAYED M. 2014. Semen cryopreservation for two local chicken strains as a tool for conservation of Egyptian local genetic resources. Egyptian Poultry Science Journal 34(2): 607–618.

SHARMA A, SOOD P, CHAUDHARY JK. 2020. Effect of different concentrations of glycerol in cryopreservation of gaddi goat semen. Journal of Veterinary Andrology 5(1): 01–06.

ŠICHTAŘ J, ŠIMONÍK O, FOLKOVÁ P, DOKOUPILOVÁ A, RAJMON R, SVOBODOVÁ I, JEBAVÝ L. 2016. The quality of frozen-thawed canine semen with respect to semen extender composition and sequence of ejaculate collection in dogs. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis 64(1): 169–175.

SIKARWAR AKS, RAMACHANDRAN N, RANJAN R, GANGWAR C, AGRAWAL JK. 2015. Effect of different levels of glycerol and DMSO on freezability of buck semen. Livestock Research International 3(3): 71–73.

SILVA MA, PEIXOTO GCX, LIMA GL, BEZERRA JAB, CAMPOS LB, PAIVA ALC, ... , SILVA AR. 2012. Cryopreservation of collared peccaries (*Tayassu tajacu*) semen using a powdered coconut water (ACP-116c) based extender plus various concentrations of egg yolk and glycerol. Theriogenology 78(3): 605–611.

SONTAKKE SD, UMAPATHY G, SIVARAM V, KHOLKUTE SD, SHIVAJI S. 2004. Semen characteristics, cryopreservation, and successful artificial insemination in the blue rock pigeon (*Columba livia*). Theriogenology 62(1–2): 139–153. DOI: 10.1016/j.theriogenology.2003.08.018. PMID: 15159109.

TABATABAEI S. 2010. The effect of spermatozoa number of fertility rate of chicken in artificial insemination programs. Journal of Animal and Veterinary Advances 9(12): 1717–1719.

TELNONI SP, ARIFIANTINI RI, YUSUF TL, DARWATI S. 2017. SK Kedu semen cryopreservation in Beltsville Poultry Semen extender and lactated ringer's-egg yolk extender using dimethyl sulfoxide. Asian J. Poult. Sci 11: 14–19.

VILLAVERDE AISB, FIORATTI EG, PENITENTI M, IKOMA MR, TSUNEMI MH, PAPA FO, LOPES MD. 2013. Cryoprotective effect of different glycerol concentrations on domestic cat spermatozoa. Theriogenology 80(7): 730–737.