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Effects of Commercial and Homemade Extenders on Post-thaw Sperm Quality and Fertility of Semen from Ethiopian Indigenous Horro Chicken Breed

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ABSTRACT

Cryopreservation of spermatozoa represents an important strategy for *in vitro* programs designed for the conservation of the genetic material of livestock populations. The objective of this study was to evaluate the effects of homemade tris-egg yolk-based and commercial poultry semen extenders on post-thaw sperm quality, fertility, and hatchability of semen from the Ethiopian Indigenous Horro chicken breed. A total of 30 roosters were used for semen collection, and 160 adult hens were inseminated artificially. The collected, qualified, and pooled semen samples were divided into three groups. Each semen sample was diluted with a homemade tris-egg yolk-based extender, Dimethyl-formamide commercial extender, and Commercial Beltsville Poultry Extender. Each extended semen was further divided into 20 sterile tubes as replicates. The extended semen samples were cryopreserved in liquid nitrogen using standard procedures. Changes in post-thaw spermatozoa mass and progressive motility, *in vitro* viability, morphological abnormality, fertility, and hatchability were evaluated. The post-thaw evaluation showed a decrease in the mass and progressive motility, morphologically normal spermatozoa, and an increase in dead spermatozoa and spermatozoa with bent necks, compared to fresh semen. There were significant differences in progressive sperm motility, motility, and *in vitro* viability between commercial and homemade cryoprotectants. However, no significant difference was observed in mass motility across the extenders. The commercial Dimethyl-formamide extender was found to be the most suitable regarding the proportion of morphologically normal sperm and *in vitro* viability rate of cryopreserved sperm samples. There were no significant differences across all treatments in terms of fertility and hatchability rate. However, there was a significant difference between the control treatment and commercial extenders in terms of fertility and hatchability. The findings indicated favorable outcomes for a tris-egg yolk-based extender that was prepared locally with regard to the cryopreservation of poultry semen. Additional investigations are recommended to enhance the fertility and hatchability of the semen.

Keywords: Cryopreservation, Horro, *In vitro* viability, Morphology, Motility, Semen, Sperm

INTRODUCTION

Cryopreservation of semen is an efficient method for the *ex-situ* management of avian genetic resources (Ehling et al., 2012). However, the success of cryopreservation largely depends on the choice of semen extender used. Therefore, evaluating and identifying the most suitable semen extender for the cryopreservation of semen from the Ethiopian Horro chicken breed is essential to ensure the preservation of its genetic resources. Extenders can be defined as buffered salt solutions used to prolong the viability of good-quality semen. The main advantage of commercial extenders is their availability as well as standardized composition and application (Petriˇcáková et al., 2022). Cryopreservation of spermatozoa could play a crucial role in genetic resource conservation as the conservation of poultry genetic resources by the living flock is costly (Han et al., 2005).

Many factors, such as the different types of cryoprotectants (CPAs), packaging, and cooling rates, could affect the quality of cryopreserved semen (Gerzilov, 2010). Glycerol is the most widely used cryoprotectant for cryopreservation of chicken semen. Continuous studies and improvements in the use of glycerol in semen extenders may enhance the fertility of post-thawed semen (Zong et al., 2022). Practices for *ex-situ* preservation of endangered breeds have been studied for the past decades (Thélie et al., 2019). Genetic stocks of chicken genetic diversity in cryobanks have been developed using cryopreservation of semen and primordial germ cells, and gonadal tissues. Due to its non-invasive nature, the cryopreservation of semen stays the preferred method (Thélie et al., 2019). Several protocols have been developed to cryopreserve semen from chickens. However, the results obtained gave a highly variable success rate. Several studies have reported that Glycerol-based CPAs maintain the quality of spermatozoa (Seigneurin and Blesbois, 1995; Partyka et al., 2012; Miranda et al., 2017). Therefore, this study aimed to assess post-thaw microscopic qualities and fertility of cryopreserved semen using locally prepared Glycerol-based CPA and commercial CPAs of semen

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collected from Horro chickens.

MATERIAL AND METHODS

Ethical approval

The present study followed institutional guidelines for humane animal treatment and complied with relevant legislation from Addis Ababa University College of Veterinary Medicine, Bishoftu, Ethiopia.

Animal management

For semen collection, a total of 30 adult Horro cockerels with an average age of 40 weeks and an average body weight of 1.7 kg were used. The roosters were kept separately from the hens and trained for semen collection by abdominal massage technique for 2 weeks. For artificial insemination, 160 adult hens with similar age groups were used. The experimental chickens were purchased from Debrezeit Agricultural Research Center, Bishoftu, Ethiopia. The roosters and the hens were kept in a deep litter system with a depth of 12 cm in pens that had a total area of 30 m^2. They were fed a breeder ration containing 17% crude protein and 2800 Kcal/Kg energy. Feed was provided twice a day at an amount of 110 gm/bird/day and water was provided *ad libitum*. All experimental chickens were vaccinated for major diseases, including Newcastle, Marek's, Gumboro, fowl pox, and fowl typhoid. The chickens were given a 2-week adaptation period in the experimental environment before sample collection and artificial insemination.

The average temperature and humidity of the chicken house were 22°C and 41%, respectively. The lighting conditions used in the experiment were 16 hours for the hens during the laying period and 12 hours for the roosters.

Extender preparation

The homemade extender used in this study was a Glycerolized tris-egg-yolk-based extender. Semen diluents were prepared by mixing tris (base), citric acid, fructose, and chicken egg yolk. Antibiotic 25 mg of gentamicin (Wockhardt Ltd, UK) was added into the extender at room temperature of 25°C. The composition of diluents is presented in Table 3.

Table 1. Breeder ration formula used during the experiment

Feed ingredient	Inclusion rate (%)
Corn	52
Soy cake	10
Meat and bone meal	6
Wheat bran	15
Noug cake	9
Limestone	6
Breeder premix	0.5
Lysine	0.1
Methionine	0.1
Molasses	1
Salt	0.3

Breeder premix: Industrial, well-balanced premix that ensures fertile, hatching eggs and ultimately strong chicks. It contains vitamins and minerals.

Table 2. Vaccination schedule used for Horro chicken

Age	Vaccination against	Application
Day 1	Marek	Subcutaneous (neck)
Day 2	Newcastle disease	Eye drop
Day 7	Gumboro	Drinking water
Day 14	Newcastle (Lasota)	Drinking water
Day 18	Gumboro	Drinking water
Week 6	Newcastle (Lasota)	Drinking water
Week 8	Fowl typhoid	Injection
Week 9	Deworming	Drinking water
Week 10	Fowl pox	Wing stab
Week 14	Fowl typhoid	Injection

The vaccines originated from the National Veterinary Institute, Bishoftu, Ethiopia.

Table 3. Contents of the homemade extender

Contents	Amount
Tris (base)	2.42 gm
Citric acid	1.48 gm
Fructose	4 gm
Chicken egg-yolk	20 % v/v
Gentamicin	200k IU
Double distilled water	100 ml

pH was adjusted to 6.7

Semen collection and initial evaluation

Semen was collected using the Quinn and Burrows abdominal massage technique developed. The semen was collected with a sterile tube. Two ejaculates were collected from the roosters at Debre Zeit Agricultural Research Center, Poultry Farm, Bishoftu, Ethiopia. After collection, the semen was maintained in a water bath at 37°C and subjected to pre-freeze evaluation

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on site. Fresh semen collected was evaluated for semen volume, color, pH, sperm concentration (bill/ml), motility (%), morphological abnormality (%), and live percent. Qualifying ejaculates were pooled to get sufficient semen for a replicate having motility > 60%, live percent > 70%, and morphological abnormality < 30% for further processing (Getachew et al., 2015).

Semen processing for cryopreservation

Qualifying ejaculates (pearly-white, free of any fecal contamination, above 0.3 ml, sperm motility of above 60%, sperm concentration of above 1 × 109 sperm cells/ml) were used for cryopreservation. The pooled semen was divided into three equal volumes and diluted with E1 (homemade extender), E2 (commercial extender Avian Semen Diluent, Minitube International, Tiefenbach, Germany), and E3 (commercial extender Beltsville Poultry Semen Extender, P2-7450, continental, Delavan, WI, USA) added at a ratio of 1:3 (semen: extender). The osmolarity of the locally prepared extender used for the cryopreservation was 320 mOsmol/kg and pH of 6.7. The diluted semen was distributed equally in 60 sterile glass tubes each. The experimental layout is presented in Table 4.

The CPAs were supplemented to each tube 1:5 v/v as a final concentration. The semen samples were equilibrated in a refrigerator at 5°C for 40 minutes (Silyukova et al., 2022). Equilibration is a process that helps the spermatozoa become more resistant to the effects of cold shock. During equilibration, the spermatozoa are permeated with glycerol, which allows for the establishment of ionic and osmotic equilibrium with the surrounding media. This equilibrium helps protect the spermatozoa during the cryopreservation process. The freezing procedure was followed by the static vapor freezing method. Sample freezing started by placing the tubes on racks in a grill wide-mouthed liquid nitrogen container kept 32 cm away from the brim (mouth) of the container. After vapor freezing, the straws were collected and plunged into pre-cooled goblets for storage. For the post-thaw evaluation, the sperm samples were thawed after 7 days by keeping the sperm in the air for 90 seconds and then in the water bath of 37°C for another 60 seconds. The mass sperm motility, progressive sperm motility, morphological abnormalities, *in vitro* viability, and acrosome integrity of the frozen semen were evaluated following Gerzilov (2010).

Table 4. Experimental groups for post-thaw sperm quality analysis

Treatment	Type of extender	Number of replications
E1	Semen diluted with homemade extender	20
E2	Semen Diluted with DMF extender	20
E3	Semen diluted with Beltsville PSE	20

E: Extender, DMF: Dimethyl-formamide, PSE: Poultry semen extender

Post-thaw semen quality assays

Semen was evaluated based on ejaculate volume, color, and concentration using a standard hemocytometer, motility, viability, and morphology percentage of spermatozoa. Mass and progressive sperm motility were assessed microscopically (400×) by putting a drop of semen on the slide. For morphological evaluation, semen was mixed with 1.6% eosin and 6% nigrosine and observed under a light microscope (×1000 magnification, MSC-P200, China) under oil immersion. A total of 200 spermatozoa were examined to determine the percentage of abnormal sperm using the Eosin-nigrosine stain. The stain was applied at a magnification of 1000X to assess the *in vitro* viability of the spermatozoa (Zong et al., 2022).

Artificial insemination and fertility evaluation

For this purpose, a total of 160 adult hens with similar age groups (35-40 weeks of age) were used. Forty hens were used in each treatment (Table 5). The hens were divided into four pens, each pen containing 10 hens, and kept for 20 days without exposure to males. Each extender and fresh semen were inseminated for fertility evaluation. The insemination was performed during the afternoon since during the morning, most hens have an egg in their oviducts, thus obstructing the free passage of semen to the ovary. A volume of 0.3 ml of thawed semen was inseminated at a 7-day interval over three weeks. The vaginal artificial insemination was performed using a 1 ml capacity sterile syringe (Getachew et al., 2015). A total of 400 eggs, 100 eggs from each treatment were collected to analyze fertility in the current study. Hens in each treatment were divided into three pens containing 40 hens each as replications. Finally, 25 eggs were collected from each pen. Hatched eggs were collected in the morning. Then, uncracked and clean eggs of at least 50 g were marked and identified by pen number and treatment number, stored sharp point of the egg downward, and pre-heated for 12 hours at 25°C prior to incubation. The eggs were placed randomly in racks and trays, specifically in 150-egg capacity trays at the ELERE farms hatchery unit. These trays were then subjected to incubation for a period of 18 days at a temperature of 37.5°C and a relative humidity of 60-70%. To ensure uniform development, the eggs were turned every hour at 90° during the incubation period. All eggs were candled individually on day 18 of incubation. Clear eggs were removed, opened, and inspected for evidence of embryo development. In the absence of an embryo, eggs were classified as infertile. Fertility and hatchability were calculated according to the following formula.

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Fertility (%) = $\frac{\text{Number of eggs fertile}}{\text{Number of eggs set}} \times 100$

Hatchability (%) = $\frac{\text{Number of eggs hatched}}{\text{Number of Eggs fertile}} \times 100$

Table 5. Experimental groups for fertility and hatchability analysis

Treatment	Type of extender	Number of hens	Number of pens	Number of hens in each pen	Number of eggs collected for incubation
E1	Semen stored using a homemade extender	40	4	10	100
E2	Semen stored using DMF extender	40	4	10	100
E3	Semen stored using Beltsville PSE	40	4	10	100
E4	Fresh semen (control)	40	4	10	100

E: Extender, DMF: Dimethyl-formamide, PSE: Poultry semen extender

Statistical analysis

The data collected during the study period were subjected to Analysis of Variance (ANOVA) using the latest version of STATA, version 12. The data on semen quality parameters were analyzed using ANOVA. When F-test is found significant, means were compared using LSD. The p-value less than 0.05 was considered to determine a statistically significant difference ($p < 0.05$). A one-way completely randomized design was utilized to evaluate the effect of CPAs on sperm quality assays.

RESULTS

Fresh semen characteristics

A summary of the results of semen characteristics addressed in this study is presented in Table 6.

Table 6. General semen characteristics of the Horro chicken breed

Semen characteristics	Mean semen characteristics
Ejaculate volume (ml)	0.36
Color	Milky white
Texture	Moderate viscous
Sperm total concentration/ml	$5.5X10^9$
Sperm count/ejaculate	$1.98X10^9$
Ph	7.2

Effect of cryoprotectants on sperm quality

The effect of CPAs on the sperm quality of Horro chicken breed is presented in Table 7. There were no significant differences in mass motility across CPAs ($p > 0.05$). However, there were significant differences between homemade and commercial extenders regarding progressive sperm motility, motility, and *in vitro* viability ($p < 0.05$). Regarding the commercial extender, there were no significant differences in all sperm quality parameters ($p < 0.05$). The semen preserved using commercial extenders indicated a significantly higher morphologically normal sperm and *in vitro* viability rate, compared to the homemade extender ($p < 0.05$). Percent progressive motility recorded the same for Dimethyl-formamide and Beltsville Poultry semen extender.

Effect of cryoprotectants on fertility and hatchability

Fertility and hatchability data are presented in Table 8. There were no significant differences across all extenders in terms of fertility and hatchability rate ($p > 0.05$). However, a significant difference was observed between the fresh semen inseminated (control treatment) and cryopreserved semen using extenders ($p < 0.05$).

Table 7. Effect cryoprotectants on post-thaw sperm quality of Horro breed

Factors \ Mean ± SE Sperm Parameters	Mass motility (%)	Progressive motility (%)	Morphologically normal (%)	Viability (%)
Homemade extender	48.5 ± 1.5^a	23.75 ± 0.81^b	55.25 ± 1.11^b	33.2 ± 0.96^b
DMF	51 ± 1.52^a	28.2 ± 0.56^a	64.25 ± 0.91^a	42.75 ± 0.73^a
Beltsville PSE	51.5 ± 1.31^a	28.2 ± 0.57^a	62.05 ± 0.70^a	42.15 ± 0.52^a

SE: Standard error, DMF: Dimethyl-formamide, PSE: Poultry semen extender, [ab]: Different superscript letters within the same column show significant differences among the groups ($p < 0.05$).

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Table 8. Effect of cryoprotectants on fertility and hatchability of Horro breed sperm

Factor \ Treatment	Homemade extender	DMF	Beltsville PSE	Control (Fresh semen inseminated)
Fertility rate (%)	41 ± 1.82[b]	48 ± 2.27[b]	46.25 ± 1.31[b]	87.93 ± 1.64[a]
Hatchability rate (%)	77.23 ± 2.25[b]	80.25 ± 1.31[b]	78 ± 2.68[b]	87.25 ± 1.03[a]

DMF: Dimethyl-formamide, PSE: Poultry semen extender, [ab]: Different superscript letters within the same row show significant differences among the groups ($p < 0.05$).

DISCUSSION

Semen color depend on the species of roosters used. However, the milky white color of the semen observed in this current study is consistent with previous reports (Hafez and Hafez, 2000; Peters et al., 2008; Mussa et al., 2023). The color of domestic fowl semen varies from a dense opaque suspension to a watery fluid secreted by various reproductive glands, from a relatively high sperm density or degrees of clear to milky white, with increased sperm numbers (Hafez and Hafez, 2000). According to Gordon (2005), the average ejaculate volume of semen using the abdominal massage technique was 0.25 ml. Bah et al. (2001) also reported an ejaculate volume of 0.28 ml in Nigerian local cocks. Cole and Cupps (1977) reported ejaculate volume within the range of 0.1-1.5 ml per ejaculation using abdominal massage techniques. Moreover, Hafez and Hafez (2000) indicated that the average sperm volume collected from white leghorn varied from 0.2 to 0.5 ml. These studies are in agreement with the result found in the current study which was 0.36 ml/ejaculate.

The average sperm concentration in the present study was $5.5X10^9$ ml (Table 6). Results of studies performed by David et al. (2015) and AL-Saeedi et al. (2019) indicated the concentration of sperm ranging $3.4\text{-}6.8 \times 10^9$ ml. According to Gordon (2005), the average sperm concentration is 5000 × 106 sperm/ml. The sperm concentration recorded in the present study is within the range reported by Hafez and Hafez (2000), that is $3000\text{-}7000 \times 10^6$ spermatozoa/ml. The sperm concentration is attributed to the alkaline nature of the accessory sex gland fluid, as reported by Bah et al. (2001) and Peters et al. (2008). The use of preserved semen in poultry production is markedly less than in mammals due to the low resistance of poultry spermatozoa to heat shock, leading to a reduction of the fertility of thawed semen (Andreea and Stela, 2010; Partyka and Niżański, 2022). Poultry sperm are more susceptible to damage caused by extreme heat changes compared to mammalian sperm. This increased sensitivity is attributed to the higher levels of polyunsaturated fatty acids present in poultry sperm (Bréque et al., 2003). Despite the implementation of various protocols in cryopreservation to prevent damage to sperm, the viability of post-thaw sperm is still not satisfactory (Bacon et al., 1986; Gliozzi et al., 2011). Therefore, it is recommended to develop strategies that can reduce these structural and biochemical damages.

An evaluation of the extenders on quality of cryopreserved Ethiopian indigenous chicken semen showed that Dimethyl Formamide extender yielded a higher progressive motility percentage (28.2 ± 0.56), *in vitro* viability percentage (42.75 ± 0.73), and morphologically normal sperm percentage (64.25 ± 0.91), compared to other treatments ($p < 0.05$, Table 3). These results were similar to those reported by Łukaszewicz et al. (2008), indicating that the egg yolk and sodium citrate extender developed by Lukaszewicz (2002) yielded better results in gander semen. This result is supported by the report of Christensen (1995) in which the sperm quality attributes are highly affected by CPAs and osmolarity.

CONCLUSION

Post-thaw quality of sperm is highly dependent on the use of appropriate semen extender and freezing procedures. In this study, Dimethyl-formamide extender demonstrated better results in all sperm quality parameters, except for mass motility, when compared to the homemade extender and commercial Beltsville PSE. However, it is important to note that the overall sperm quality and fertility outcomes were still lower compared to those observed in mammalian species. Further studies are recommended to develop standard freezing procedures and the use of cryoprotectants. Based on the results obtained regarding sperm quality and fertility rates, it is concluded that poultry semen cryopreservation is more suitable for establishing a semen biobank rather than for commercial use. However, further studies are needed to identify more effective procedures and cryoprotectants that can enhance post-thaw sperm quality even further.

DECLARATIONS

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Authors' contributions

Tarekegn Getachew, Gebeyehu Goshu and Alemayehu Lemma designed the experiments and Tarekegn Getachew performed the experiments. Tarekegn Getachew derived the models and analyzed the data. Gebeyehu Goshu and Alemayehu Lemma assisted with standardizing data collection and data analysis. Tarekegn Getachew wrote the manuscript in consultation with Gebeyehu Goshu and Alemayehu Lemma. All authors read and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors have declared that no competing interest exists

Ethical consideration

All ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all authors.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author (T. Getachew), upon reasonable request.

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