Original Article

Sahel Journal of Veterinary Sciences

Sahel J. Vet. Sci. Vol. 18, No. 4, pp 29-34 (2021)

Article History
Received: 05-08-2021
Revised: 30-12-2021
Accepted: 30-12-2021
Published: 31-12-2021

Effects of Egg Yolk and Coconut Milk Based Extenders on the Qualities of Chilled and Cryopreserved Turkey (*Meleagris gallopavo*) Semen in Maiduguri, Nigeria

***[1]Asuku, S. O., [1]Alkali, I. M., [1]Mustapha, A. R., [1]Bukar, M. M., [1]Waziri, M. A., [1]Amin, J. D. and [2]Ribadu, A. Y.**

[1]Department of Theriogenology, Faculty of Veterinary Medicine, University of Maiduguri, Nigeria
[2]Department of Theriogenology, Faculty of Veterinary Medicine, University of Abuja

* *Author for Correspondence: suleimanomeiza06@gmail.com*

ABSTRACT

This study investigated the effects of egg yolk and coconut milk-based extenders on the motility and morphology of chilled and post thawed turkey *(Meleagris gallopavo)* semen in Maiduguri, Nigeria. A total of 112 semen samples were collected from seven matured turkey toms, twice weekly for 2 months using the dorso-abdominal massage method. Freshly collected semen were pooled, divided into three aliquots and diluted with modified Ringer's buffer, egg yolk and coconut milk-based extenders, respectively. An aliquot of each extended semen was stored at 37°C and 5°C, and then evaluated hourly for 24 hours. Extended semen samples were also cryopreserved in liquid nitrogen and post thaw qualities were assessed after 24 hours. The progressive motility of spermatozoa was maintained for 6 hours at 37°C and up to 12 hours at 5°C when preserved in coconut milk and egg yolk-based extenders. It was found that the use of 1% coconut milk or 1% egg yolk-based extenders improved the post thaw quality of turkey semen once frozen at -196°C. It was also observed that the coconut milk-based extender preserved the spermatozoa motility and reduced sperm abnormalities better than the egg yolk-based extender.

Keywords: Modified Ringers, Egg Yolk, Coconut Milk, Cryopreservation, Turkey semen

INTRODUCTION

Turkey production is one of the fastest growing agricultural sub-sectors, substantially reared to improve food security, nutritional health and poverty alleviation especially in developing countries (Mottet and Tempio, 2017). In Nigeria, second to chicken production, turkey *(Meleagris gallopavo)* domestication is increasingly gaining momentum among peasant farmers. Turkey breeding relies almost entirely on Artificial Insemination (AI) and for the industry to benefit from the modern reproductive technique (AI), emphasis on good quality semen during preservations is critical (Dumpala *et al.,* 2006). However, there is a lack of large-scale turkey production in the country which may be connected to lack of turkey semen cryo-banks and effective avian artificial insemination units required to encourage interested turkey breeders and improved production.

Turkey semen rapidly loses fertility potential index within few hours of in-vitro storage, hence a need for its immediate preservation (Iaffaldano *et al.,* 2005). Temperature changes, osmotic stress and storage time are important factors implicated in the deterioration (Dumpala *et al.,* 2006; Swain and Smith, 2010). In order to sustain the fertilizing capability of in-vitro stored spermatozoa, semen is stored at 2 – 8°C (Donoghue and Wishart, 2000) and is preserved in appropriate extenders (Iaffaldano *et al.,* 2005). Good extenders provide energy sources (fructose or glucose), oxygen and optimal pH for sperm survival during storage (Akcay *et al.,* 2006; Siudzinska and Lukaszewicz, 2008). The Beltsville turkey semen extender has been developed and reported as the most frequently used turkey semen extender; nonetheless, they are expensive and not readily available in most developing countries like Nigeria. The extension and storage of semen also allows the efficient use of the characteristic low ejaculates produced by turkey toms (Dumpala *et al.,* 2006).

Presently, chilling (5°C) and cryopreservation (-196°C) are two basic technologies reported for turkey semen storage (Iaffaldano *et al.,* 2016) although in chilling, the semen qualities are rarely preserved for longer than 6 hours without irreversible loses in its fertilizing capability (Iaffaldano *et al.,* 2005). Choice of cryoprotectants (CPAs) and temperature transition are also key factors to determine sperm survivals following freezing protocols (Blesbois, 2007; Iaffaldano *et al.,* 2016). Different cryoprotectants (CPAs) have been used individually and in combination (Long *et al.,* 2014) but very

little information is available with regards the best cryoprotectant for turkey semen storage (Kuzlu and Taskin, 2017). Non permeable CPAs are generally recommended since it minimizes cryo-damages (ice crystals formation) observed with the permeable CPAs (Iaffaldano *et al.,* 2016).

Coconut milk and egg yolk are known products utilized in varying combination and proportion as components of semen extenders. Patil *et al.* (2017) described coconut milk as liquid extract obtained from a grated coconut meat. The milk contains energy substrates (glucose, fructose, lactose, and sucrose), fatty acids, protein, amino acids (glutamic acid), vitamins and phospholipids (Patil *et al.,* 2017) while egg yolk contains large amounts of low-density lipids known to have cryoprotective effect on spermatozoa (Moussa *et al.,*2002). Currently, the improvement of long-term liquid storage is still considered very important since the commercial production of turkeys relies almost entirely on artificial insemination (Iaffaldano *et al.,* 2005). As part of efforts in improving storage regimens for turkey semen preservations, this study aimed to provide information on the suitability of egg yolk and coconut milk-based extenders on the semen qualities of chilled and cryopreserved turkey semen in Nigeria.

MATERIALS AND METHODS

Experimental Animals and Feeding Management

Seven (n = 7) matured turkey toms weighing approximately 12 – 15 kg were used for the study as sperm donors. They were purchased from poultry breeders in Maiduguri and housed in individual cages at the Faculty of Veterinary Medicine Large Animal Clinic Complex, University of Maiduguri. They were acclimatized and protected from effects of low temperature and high air velocity to maintain good welfare and behavior (Mendes *et al.,* 2020). They were fed with a commercial poultry feed (Vital® feed) containing 125g/kg crude protein. Clean water was given to them *ad libitum.*

Preparation of the Buffer and Extenders

The buffer was the Modified Ringer's Buffer Solution prepared as described by Hafez (2000). The egg yolk preparation was according to the method described by Balogun *et al.* (2017). For the coconut milk, grated coconut, together with the coconut water was homogenized, filtered thrice and transferred into clean centrifuge tubes and centrifuged at 4000 rpm (revolutions per minute) for 10 minutes. The coconut milk was obtained as the clear fluid below the supernatant. The milk was aspirated, re-centrifuged and the final aspirate used for the semen extension. A solution of the Modified Ringer's Buffer +1% coconut milk + 1 % glucose constituted the coconut milk based extender while a solution of the Modified Ringer's Buffer + 1% chicken egg yolk + 1 % glucose was the egg yolk based extender.

Semen Collection, Extension, Storage and Evaluation of the Turkey Semen

Method of semen collection was according to Burrows and Quinn (1937) and collections were made twice a week (Mondays and Thursdays) for 8 consecutive weeks. Freshly collected semen were pooled and divided into three aliquots in graduated 1ml Eppendorf tubes. One aliquot was diluted in the Modified Ringers Buffer while the remaining two aliquots were diluted in a ratio 1:3 (1 part semen: 3 parts extender) with egg yolk and coconut milk-based diluents respectively. Each of the diluted semen was further divided into two fractions; one fraction was stored at 37°C in a water bath and the other was chilled to 5°C in a Hisense® refrigerator (Model RS230S). The temperature was monitored with a digital laboratory thermometer (Thermo-fisher® India) and thereafter, progressive motility and morphologic abnormalities were evaluated hourly for 6 hours, at 12 hours and at 24 hours post extension.

Freezing and Thawing of the Semen

Prior to semen collection, the coconut milk and egg yolk-based extenders with 10% glycerol were prepared in advance, measured in a graduated Eppendorf tube and stored at 15°C before use. Progressive motility and morphologic abnormalities were evaluated immediately after the collection and extension. Extended samples were loaded in plastic straws (0.25ml), sealed with a polyvinyl straw sealant and cooled to 8°C for 10 minutes. Equilibration was at 5°C for 10 minutes, and liquid vaporization at -140°C for 10 minutes (placed on a rack 4cm above the liquid nitrogen) (Long *et al.,* 2014). The straws were stored in liquid nitrogen (-196°C) and thawing was done at 8°C for 10 seconds. The post thaw qualities were assessed after 24 hours of storage.

Statistical Analyses

Data obtained was subjected to repeated measures Analysis of Variance (ANOVA) using IBM® SPSS® statistical software (version 20). Results were summarized and expressed using descriptive statistic (Mean ± Standard deviation) and values at a significance level of $p < 0.05$ were considered statistically significant.

Ethical Statement

The ethics governing the use and conduct of experiments on animals were strictly observed, and the experimental protocol was approved by the Research and Ethics Committee, University of Maiduguri.

RESULTS

Progressive Motility (%) of Spermatozoa in Extended Turkey Semen Stored at 37°C

Compared with the coconut milk and egg yolk-based extenders, semen samples diluted with modified ringer's solution showed the highest progressive motility (91.5 ± 3.5) immediately after dilution. The quality decreased significantly ($p < 0.05$) to 9.2 ± 4.4 % when assessed 4 hours later at 37°C (Table 1). Although, the progressive motility was higher in the modified ringer's solution within 2 hours of the semen storage, coconut milk and egg yolk-based extenders maintained a significantly ($p < 0.05$) higher motility compared with the modified Ringers from 3 hours and beyond. No spermatozoon was motile at 6 hours of semen dilution in modified ringer's solution but greater than 18% motility was observed in coconut milk and the egg yolk-based semen extenders.

Progressive Motility (%) of Spermatozoa in Extended Turkey Semen Chilled to 5°C

The highest progressive motility (92.8 ± 5.6 %) was recorded in modified ringer's buffer solution immediately after collection, but later decreased significantly ($p < 0.05$) to 2.8 ± 2.6 % after semen was chilled to 5°C and maintained for 24 hours. There was no significant decrease in the motility within two hours of semen dilution in any of the diluents. However, by 3 to 24 hours, coconut milk and egg yolk-based extenders maintained the progressive motility higher than in modified ringer's buffer alone. Over 40 % motility was observed in coconut milk and egg yolk-based extenders at 12 hours compared with the 12.5 % motility observed in the modified ringer's solution (Table 2).

Morphologic Abnormalities (%) in Extended Turkey Semen Chilled to 5°C

Immediate assessment of semen after collection showed the highest morphologic defects (4.1 ± 1.3 %) in modified ringer's solution. These abnormalities increased significantly ($p < 0.05$) to 17.1 ± 2.8% after dilution and chilling to 5°C for 24 hours. Morphologic defects were lower in coconut milk and egg yolk-based extenders than in the buffered medium, although this became only significant at 12 to 24 hours post extension (Table 3). The least abnormalities were observed in the coconut milk-based extender.

Pre and Post Thaw Progressive Sperm Motility (%) in Extended Turkey Semen Frozen at -196°C

The highest progressive motility (76.4 ± 4.8 %) was observed in modified ringer's buffer solution during pre-freezing evaluation at 15°C (Table 4). Although, the motility was significantly better ($p < 0.05$) in coconut milk-based extender (40.0 ± 1.2) than in the egg yolk based (32.7 ± 2.8), both extension media showed a significantly higher ($p < 0.05$) post thaw motility than the modified Ringer's buffer solution.

Post Thaw Sperm Morphologic Defects (%) in Extended Turkey Semen Frozen at -196°C

The highest post thaw morphologic defect (38.4 ± 3.2) was observed in samples diluted with modified Ringer's buffer while the coconut milk-based extender maintained the lowest abnormalities (24.5 ± 2.7) as presented in Figure 1.

DISCUSSION

Progressive forward motility of spermatozoa is the most reliable quality indicator of good semen and is significant in selection of sperm donors for breeding purpose. In this study, the higher progressive motility observed in coconut milk and egg yolk-based extenders, when compared with the modified ringer's buffer solution, suggests the positive impact of the exogenous glucose that was incorporated into the extenders and perhaps the buffering components found in coconut milk and the chicken egg yolk. Extenders are known to enhance provision of energy substrates, adequate osmotic pressure and suitable pH for improved sperm survivability. This observation is in accordance with a finding of Ackay et al. (2006) in whose work was reported, that extenders with iso-osmolar glucose constituent yields high percentage of motile spermatozoa in turkey semen after cold storage.

The quality parameters better maintained at 5°C than at 37°C especially from 4 hours and beyond may be attributed to the effect of reduced temperature on the sperm metabolism, similar to other reports made in buck semen extension by Olurode and Ajala (2016). In a previous work by Aboagla and Terada (2003), it was reported that reduced temperature minimizes metabolic process of spermatozoa and then results in rapid utilization of nutrients (fructose) by the sperm cells. Thus, the decreased motile spermatozoa observed in the current study with longer storage time, reflects the gradual depletion of nutrients required for the sperm survival. A decrease in morphologically normal and live spermatozoa over time was also observed with a corresponding increase in dead spermatozoa. This finding agrees with earlier reports by Siudzinska and Lukaszewicz (2008). In addition, the morphologic defects observed in the current study were similar to those reported by Alkan et al. (2002) and were mostly secondary abnormalities which might have occurred during collection and processing of the semen.

It was also found, that post thaw progressive motility and the morphologic abnormalities were maintained significantly ($p < 0.05$) better in the coconut milk and egg yolk-based extenders compared with the modified ringer's solution. However, the post thaw motility obtained in both coconut milk and the egg yolk-based extenders in the current study were lower than the average motility (43.3 ± 1.6 %) reported by Kuzlu and Taskin (2017) who used 5% dimethyl sulphoxide (DMSO) as cryoprotectant. The better post thaw quality of turkey semen observed in the coconut milk compared to egg yolk-based extender and the modified ringer's buffer solution may be due to presence of carbohydrate (glucose), glutamic acid, arginine, lysine and aspartic acid found in coconut milk. Previous studies showed that carbohydrate is essential component that ensures energy supplement to sperm cells and amino acids such as glutamic acid, arginine and lysine supports motility, osmotic balance and survival of spermatozoa during freezing (Iaffaldano *et al.,* 2005).

In the present study, significantly higher motility was observed in post-thawed semen extended with 1% coconut milk compared with the egg yolk-based extender. Santiago-Moreno et al. (2019) investigated the effects of seminal plasma (which contains large amounts of amino acids) on cryoresistance of chicken semen and found that removal of seminal plasma decreased DNA fragmentation damages. However, the dynamics and interactions between the contents of the seminal plasma and the coconut milk and egg yolk which resulted in the higher motility in the coconut milk-based extender remains to be established. In the egg yolk-based extender, there is abundance low density lipids and may be responsible for the improved cryoprotective effects observed on the semen qualities when compared to the modified ringer's buffer solution. The 32.7 ± 2.8and 40.0 ± 1.2 post thaw motility in egg yolk and coconut milk extenders respectively, found in the current study, approximated the findings of Di Iorio et al. (2020) who reported post thaw motility of 35.8 ± 2.2 % and 31.4 ± 1.0% in two different extenders respectively

Table 1: Progressive Motility (%) of Spermatozoa in Extended Turkey Semen Stored at 37ºC

Semen Diluents	Post Extension Storage Time (hours)								
	Immediate	**1**	**2**	**3**	**4**	**5**	**6**	**12**	**24**
Buffer Medium **Modified Ringers** Extension Media	91.5 ± 3.5^{am}	87.4 ± 3.0^{b}	70.6 ± 5.7^{cm}	38.4 ± 5.0^{dm}	9.2 ± 4.4^{em}	0.4 ± 1.3^{fm}	0.0 ± 0.0^{fm}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}
Coconut Milk - Based Extender	88.1 ± 3.0^{an}	84.8 ± 4.3^{a}	65.3 ± 5.6^{bn}	50.6 ± 5.1^{cn}	44.3 ± 4.0^{dn}	25.6 ± 4.7^{en}	20 ± 2.5^{fn}	0.0 ±0.0^{j}	0.0 ± 0.0^{j}
Egg Yolk Based Extender	85.1 ± 4.8^{an}	84.3 ± 4.4^{a}	61.5 ± 2.3^{bn}	47.3 ± 4.4^{cn}	40.0 ± 1.8^{do}	24.0 ± 4.1^{en}	18.1 ± 3.5^{fn}	0.0 ± 0.0^{j}	0.0 ± 0.0^{j}

Values (Mean ± SD) on the same row $^{(abcdefj)}$ and same column $^{(mno)}$ with different superscripts differ significantly at $p < 0.05$

Table 2: Progressive Motility (%) of Spermatozoa in Extended Turkey Semen Chilled to 5°C

Semen Diluents	Post Extension Storage Time (hours)								
	Immediate	**1**	**2**	**3**	**4**	**5**	**6**	**12**	**24**
Buffer Medium **Modified Ringers** Extension Media	92.8 ± 5.6^{a}	88.4 ± 4.7^{a}	87.3 ± 5.4^{a}	53.8 ± 8.1^{bm}	33.1 ± 7.0^{cm}	30.0 ± 4.5^{cm}	26.3 ± 4.7^{dm}	12.5 ± 5.8^{em}	2.8 ± 2.6^{fm}
Egg yolk based Extender	88.8 ± 4.3^{a}	86.9 ± 4.8^{a}	84.7 ± 5.3^{a}	66.6 ± 6.8^{bn}	59.4 ± 4.0^{cn}	55.3 ± 3.4^{cn}	50.6 ± 3.1^{cb}	40.3 ± 2.9^{dn}	20.0 ± 4.1^{en}
Coconut Milk-Based Extender	90.6 ± 4.8^{a}	88.1 ± 4.4^{a}	85.0 ± 3.2^{a}	70.0 ± 7.5^{bn}	61.6 ± 8.1^{cn}	59.1 ± 7.8^{cn}	53.4 ± 4.7^{cn}	42.8 ± 4.1^{dn}	22.8 ± 7.5^{en}

Values (Mean ± SD) on the same row $^{(abcdef)}$ and same column $^{(mn)}$ with different superscripts differ significantly at $p < 0.05$

Table 3: Sperm Morphologic Abnormalities (%) in Extended Turkey Semen Stored at 5°C

Semen Diluents	Post Extension Storage Time (hours)								
	Immediate	**1**	**2**	**3**	**4**	**5**	**6**	**12**	**24**
Buffer Medium **Modified Ringers** Extension Media	4.1 ± 1.3	6.3 ± 3.3	6.8 ± 3.2	7.6 ± 1.3	10.1 ± 4.2	11.7 ± 5.5	14.4 ± 6.0	16.8 ± 2.5^{a}	17.1 ± 2.8^{a}
Coconut Milk-Based Extender	2.9 ± 0.9	4.1 ± 1.8	5.4 ± 1.6	6.1 ± 1.3	7.6 ± 1.6	8.9 ± 2.0	11.0 ± 3.1	13.1 ± 3.2^{b}	15.4 ± 1.9^{b}
Egg Yolk Based Extender	3.9 ± 2.0	5.2 ± 2.2	6.1 ± 2.7	7.4 ± 2.8	9.3 ± 2.3	11.3 ± 2.9	12.2 ± 2.9	13.2 ± 2.2^{b}	16.1 ± 2.5^{b}

Values (Mean ± SD) on the same column with different superscripts differ significantly at $p < 0.05$

Table 4: Pre and Post Thaw Progressive Sperm Motility (%) in Extended Turkey Semen Frozen at -196°C in Maiduguri, Nigeria

Semen Diluents	Pre-freezing	Post-thaw
Buffer Medium		
Modified Ringers	76.4 ± 4.8[a]	15.7 ± 3.5[a]
Extension Media		
Coconut Milk – Based Extender	75.0 ± 7.1[a]	40.0 ± 1.2[b]
Egg Yolk - Based Extender	68.6 ± 6.9[a]	32.7 ± 2.8[c]

Values (Mean ± SD) on the same column with different superscripts [(abc)] differ significantly at $p < 0.05$

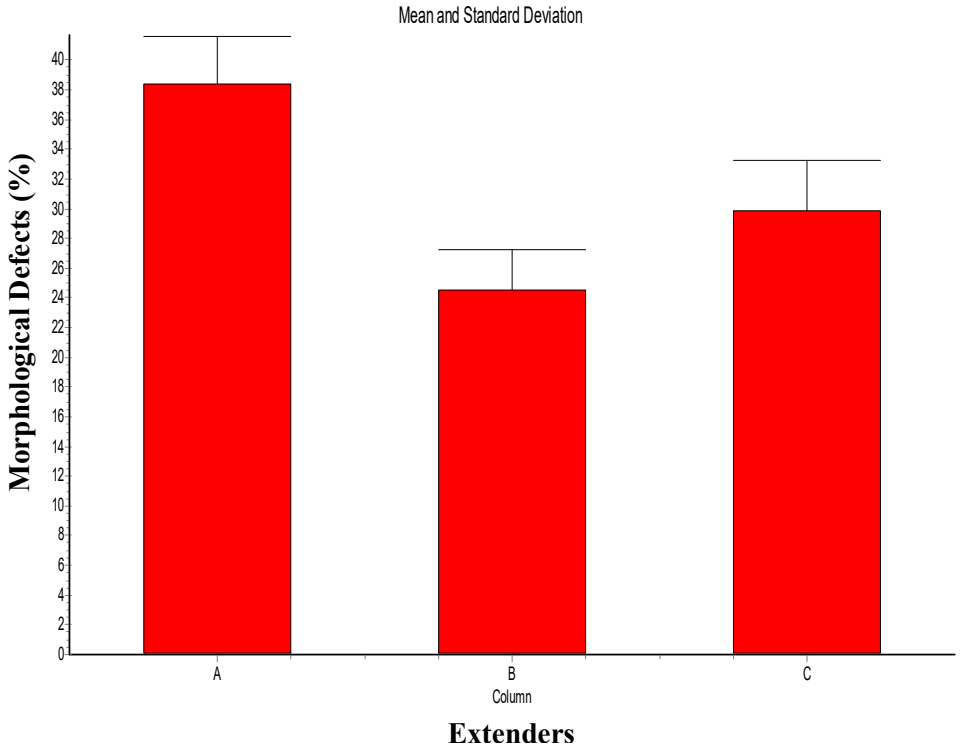

Figure 1: Post Thaw Sperm Morphologic Defects (%) in Extended Turkey Semen Frozen at -196°C
A = Modified Ringer's Buffer; B= Coconut Milk Based Extender; C= Egg Yolk Based Extender

Conclusions

Good progressive motility of spermatozoa was maintained for 6 hours at 37°C and till 12 hours at 5°C when preserved in coconut milk and egg yolk-based extenders. It was also found that use of 1% coconut milk or 1% egg yolk-based extenders, improved the post thaw turkey semen qualities when frozen at -196°C. The coconut milk-based extender preserved the spermatozoa motility and reduced abnormalities better than the egg yolk-based extender.

Acknowledgement

We wish to acknowledge the research grant from Tertiary Education Trust Fund, Nigeria that has supported this research work**.**

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant

This study was funded with a research grant from the Institutional Based Research fund, Tertiary Education Trust Fund, Nigeria, through the Centre for Research and Innovation, University of Maiduguri, Nigeria

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SOA, IMA, ARM and MMB. The draft manuscript was prepared by SOA. AYR and MAW conceived the work, read, corrected and approved the manuscript for publication. All authors read and approved the final manuscript.

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