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Evaluation of Vitamins C and E on Semen Motility and Viability in Chilled Semen of Nigerian Indigenous Turkey Toms (*Meleagris gallopavo*)

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Authors' contributions

This work was carried out in collaboration between all authors. Author WOE designed the study. Author JMM performed the statistical analysis. Author GIOK wrote the protocol. Author ESI managed the literature searches. Author OMA managed the analyses of the study. Author DSBU managed the data cleaning. Authors WOE and OMA managed the proofreading of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Oxidative stress has been established as a major cause of semen deterioration during *in-vitro* storage. The aim of this study was to evaluate the effects of supplementing the diluents of turkey semen with antioxidant vitamins C, E and a combination of vitamin C and E on semen motility and viability. Twenty healthy turkey toms aged 37-38 weeks with an average weight of 7±0.3kg were used for this study. The toms were randomly placed into four groups (T0, T1, T2 and T3) of five toms. Semen was collected bi-weekly from the turkey toms within the groups using abdominal

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massage method. Semen collected from individual turkey toms within each group were pooled, evaluated and extended with egg yolk citrate diluent containing no antioxidant (T0), 4 mg/ml Vitamin C (T1), 8 mg/ml Vitamin E (T2) and 4 mg/ml Vitamin C + 8 mg/ml Vitamin E (T3). The extended semen were packaged into biju bottles and stored at 4^0C for 48 hours. Semen motility and viability, were assessed at 0, 3, 6, 9, 12, 24 and 48 hours post dilution and storage at 4^0C. The motility and viability of the diluted semen decreased across the group with increase in storage time irrespective of the antioxidants added. T2 gave a better motility (55±2.8 %), viability (41.3± 1.5%) over a 24 hour period. The result from this study showed that supplementation of the diluents of turkey semen with antioxidants resulted in significant ($p < 0.05$) improvement in motility and viability. Vitamin E recorded a better result in relation to motility and viability. It can be concluded that the supplementation of diluents of turkey semen with antioxidant Vitamin C and E improves the shelf-live and quality of turkey semen during in-*vitro* storage.

Keywords: Meleagris gallopavo; semen deterioration; antioxidant; viability.

1. INTRODUCTION

The importance of the poultry industry in Nigeria cannot be over emphasized because of the role it plays in human nutrition and the employment opportunities it creates for her teeming population [1]. The potential of local poultry cannot be overlooked considering the huge foreign exchange implication resulting from importation of improved exotic stock and the genotype-environment interaction which leads to considerable loss of fitness of the exotic stock [2]. Nigeria is endowed with an impressive array of domestic poultry species, and they include chickens (160 million), guinea fowls (8.3 million), ducks (1.7 million) and turkeys (0.7 million) [3].

There is no known discriminatory attitude towards the production and consumption of turkeys and their products in Nigeria but that notwithstanding they are limited in population. Despite the numerous advantages of artificial insemination over natural mating in turkeys, turkey semen does not yet have the ability to survive for long period during in-vitro preservation (6-12 hours). It has been reported that the addition of antioxidant vitamins to the semen diluents possibly improved the quality of semen during in-vitro preservation. Other reports showed that supplementing the diluent of poultry semen with more than one antioxidant proved beneficial during in vitro preservation of poultry semen. The effects of different antioxidants have been extensively studied in many species including humans. Vitamin C and E appear to be two of the most extensively studied Vitamins because of their multiple beneficial applications and their availability. However, there is paucity of information comparing the effects of the various antioxidants in the preservation of turkey semen in Nigeria. Hence, a comparative study of antioxidant vitamins C, E and their combination on semen motility and viability in chilled semen of Nigerian indigenous turkey toms.

2. MATERIALS AND METHODS

2.1 Study Area

This study was carried out in the Department of Theriogenology and Production, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, situated in the Northern Guinea Savannah, between latitude 11^0 1573' N and longitude 7^0 64989' E at an elevation of 646 m above sea level. The mean annual rainfall in this area is 1,100 mm lasting from May to October. Mean daily temperature during season is 25 ^{0}C with a mean relative humidity of 72%. The dry season last from November to April, with mean daily temperature ranges of 14 – 36 ^{o}C and relative humidity of 20 – 30% [4].

2.2 Experimental Animal

Twenty (20) apparently healthy indigenous turkey aged between 37-38 weeks were used for this study. The turkey toms were sourced from local markets within Zaria. The toms were weighed, screened and treated for helminthes and blood parasites prior to the onset of the study. The toms were randomly allocated into four groups T0, T_1, T_2, and T_3 of five toms each. Turkey toms were housed individually in cages and allowed to acclimatize for a period of one month during which they were trained for semen collection. They were fed with compounded turkey grower diet containing; maize, 45%; soyabean cake, 43%; fish meal, 4%; palm oil, 3%; Bone meal, 3%; salt, 0.5% and premix, 0.5%. The feed was analysed at the Animal Nutrition Laboratory Department of Animal

Science, A.B.U, Zaria. Water was provided *ad-libitum.*

2.3 Initial Semen Collection and Evaluation

Semen was collected from the toms by abdominal massage technique as described by [5] with modification. Each tom was placed in between the arm and the body of the restrainer with its legs gently restrained. The handler firmly massages the tom's abdomen with one hand while concurrently stroking firmly the back and tail feathers with the other hand. After a few seconds, the phallus should enlarge slightly (Phallic tumescence) and the handler will move to straddle the cloaca with the thumb and forefinger of the hand situated just above the cloaca squeezing upward and inward. This action, referred to as a "cloacal stroke" will result in a partial ejaculation. It was repeated a second time to complete the ejaculation process. The semen was collected into a calibrated semen vials and evaluated for volume, colour, pH, concentration, motility (gross and individual), live-dead ratio and morphology.

2.4 Semen Evaluation

Collected semen samples were evaluated as described by [6] for:

- **Volume:** The volume was be measured immediately after collection using a calibrated tube
- **Gross motility:** Microscopic examination for wave pattern was carried out by placing a drop of raw undiluted semen on a pre-warmed glass slide then cover-slipped and viewed for motility using a microscope at x4 and x10 objective magnification.
- **Sperm concentration:** The semen concentration was determined using a haemocytometer as described by [7]. Micropipette was used to aspirate the semen to 0.5 mark and physiological saline aspirated to make up to 1.0 mark, hence diluting the semen. The pipette was wiped to remove any adhering semen. The first three drops was discarded, 2 drops was placed on either margin of the haemocytometer, cover-slipped and left for 5 minutes. It was then examined using a microscope at x40 magnification and then sperm cells counted in five squares of one chamber of the haemocytometer. The semen concentration was calculated as follows:
  Concentration (sperm cells/ml) = Average count x dilution factor $x10^4$
- **Live spermatozoa:** It was determined as described by [8]. A drop of semen sample was placed on the edge of a clean grease free glass slide and three drops of eosine-negrosine stain was mixed with the semen. A smear of the mixture was made on a clean slide.. Live viable spermatozoa have intact cell membranes which are capable of of excluding eosin dye and remain colorless. Dead spermatozoa (non-viable) on the other hand possess permeable membrane which allows the spermatozoa to pick up the eosin dye and stain pinkish. Two hundred sperm cells were counted using a microscope at x40 magnification and expressed as percentage live.
- **Sperm abnormalities:** Sperm abnormalities was determined by making a thin smear from the mixture of semen sample and eosine-negrosine on clean grease free glass slide. one hundred sperm cells was counted per slide using light microscope at x 40 magnification [8].

2.5 Diluted Semen Evaluation

2.5.1 Diluent preparation

Egg yolk citrate diluent was prepared at ambient temperature as described by [9] by measuring 2.9 grams of sodium citrate into 100 milliliters of sterile water i.e., 2.9 % w/v with the pH adjusted between 6.9 – 7.6. Fresh chicken egg was obtained and washed in warm water with mild detergent, properly rinsed and dried with a clean dry towel. To make 100 ml of the extender, 80 millilitre of sodium citrate was measured into a graduated cylinder. The egg was broken midway and the albumin discarded. The yolk was placed on a filter paper to remove as much as possible the albumin before being punctured. The yolk membrane was punctured with a sterile glass rod and the yolk was expelled into the graduated cylinder containing the extender to the 100 milliliter mark. The yolk membrane was then discarded. The measuring cylinder was inverted severally for thorough mixing. Antibiotics (penicillin G; 1000 iu/ml and streptomycin (1g/ml) was added to the mixture. The contents were shaken vigorously to ensure proper mixing.

2.6 Semen Extension and Evaluation

Semen was collected from individual turkey toms in the various groups and subjected to gross evaluation prior to pooling. Egg yolk citrate extender was prepared at room temperature, and divided into 4 parts which were supplemented with the antioxidant vitamins C (4 mg/ml), E (8 mg/ml), C+E (4 mg + 8 mg/ml), respectively, and control corresponding to groups T1, T2, T3 and T0 respectively. No antioxidant was added to the extender in the control group (T0). Semen collected from individual tom within each group was pooled, mixed and diluted with EYC extender at a dilution rate of 1:100. The diluents-semen mixture was swerved gently to facilitate proper mixing. The samples were packed into labelled Biju bottles and placed in a padded flask and then transferred into a refrigerator at 4^0C for storage.

Malondialdehyde assay, motility, viability (live-dead ratio) was measured immediately after dilution (0), at 3, 6, 9, 12, 24 and 48 hours of storage at 4^0C. The procedure was repeated trice with mean values obtained.

2.7 Data Analyses

Data collected from this study was expressed as means and their standard errors of means (SEM). One way Analysis of Variance (ANOVA) was used to compare means within and between the groups, Followed by Turkey's multiple comparison test. Values of $p < 0.05$ were considered significant. All statistical analysis was done using Graph pad prism software version 5.0.

3. RESULTS

3.1 Effects of Vitamin C, E and Their Combination on the Motility of Chilled Turkey Spermatozoa

Spermatozoa motility decreased significantly $p<0.05$ across the groups with increase in storage time regardless of the antioxidants added to the diluents as shown in Fig. 1. At 0 hour post semen dilution, spermatozoa motility were 91.7±2.9%, 81.7±2.9%, 93.3±2.9%, and 85.0±8.7% for T0, T1, T2 and T3 respectively, while at 3 hours post dilution, spermatozoa motility were 75.0±2.9%, 76.7±1.7%, 86.7±3.3%, and 71.7±1.7% for T0, T1,T2 and T3 respectively. At 6 hours post dilution, spermatozoa motility reduced to 68.3±1.7%, 70.0±0.0%, 80.0±2.9% and 65.0±2.9% for T0, T1, T2 and T3 respectively. The motility at 9 hours was 55.0±2.9%, 63.3±2.9%, 76.7±2.9% and 60.0±2.9% for T0, T1, T2 and T3 respectively. At 12 hours, the motility were 38.3±4.4%, 46.7±1.7%, 65.0±2.9% and 50.0±0.0% for T0, T1, T2 and T3 respectively. At 24 hours post dilution, spermatozoa motility reduced to 0.0±0.0%, 0.0±0.0%, 55.0±2.9% and 10.0±5.7% for T0, T1, T2 and T3, respectively. The lowest motility result was obtained at 48 hours post dilution (0.0±0.0%, 0.0±0.0%, 8.3±4.4% and 0.0±0.0% for T0, T1, T2 and T3 respectively.

3.2 Effects of Vitamins C, E and Their Combination on the Viability of Chilled Turkey Semen

The viability of diluted chilled turkey semen as represented by per cent live is shown in Figure 2. The per cent live spermatozoa at 0 hour post dilution were 94.7±0.9%, 93.0±2.5%, 92.3 ±1.4% and 90.3 ± 1.5% for T0, T1 T2 and T3 respectively. At 3 hours post dilution, the percent live spermatozoa were 81.7 ± 1.5%, 84.0 ± 0.6, 89.7 ± 0.9% and 86.3 ± 1.8% for T0, T1, T2 and T3 respectively. At 6 hours post dilution, the percent live results were 61.7 ± 1.8%, 75.0 ± 0.8, 83.0 ±1.5% and 78.0 ± 1.5% for T1, T2, T3 and T4 respectively. At 9 hours post dilution, the per cent live spermatozoa were 55.0 ±2.9%, 65.0 ± 2.6%, 71.0 ± 2.3% and 69.7 ± 4.7% for T1,T2,T3 and T4 respectively. At 12 hours post dilution, the per cent live spermatozoa were 35.3 ± 3.9%, 47.0 ±2.6%, and 55.0 ± 2.6% and 47.0 ±3.8% for T1, T2, T3, and T4 respectively. The percent live spermatozoa at 24 hours post dilution was 10.3 ± 1.2%, 19.0 ± 3.5%, 41.3 ± 1.5% and 25.0 ±4.0%. The lowest per cent live result was obtained at 48 hours post dilution (0.0 ±0.0%, 4.0 ± 0.6%, 8.3 ± 0.9% and 3.7 ± 0.9% for T1, T2, T3 and T4, respectively).

4. DISCUSSION

There was no difference in the mean semen motility between the various treatment groups and the control at 0 hours post-dilution. This was in agreement with the report of [10]. The motility results decreased across the groups with increase in storage time but the decrease in per cent motility was more drastic for the control (T0) and T1 (VIT C) which recorded a motility of 0% at 24 hours of storage. This result was in agreement with the report of [10] which stated

that the addition of vitamin C to semen diluents did not produce any difference in sperm motility over the control during the storage period of study. Other authors reported a similar

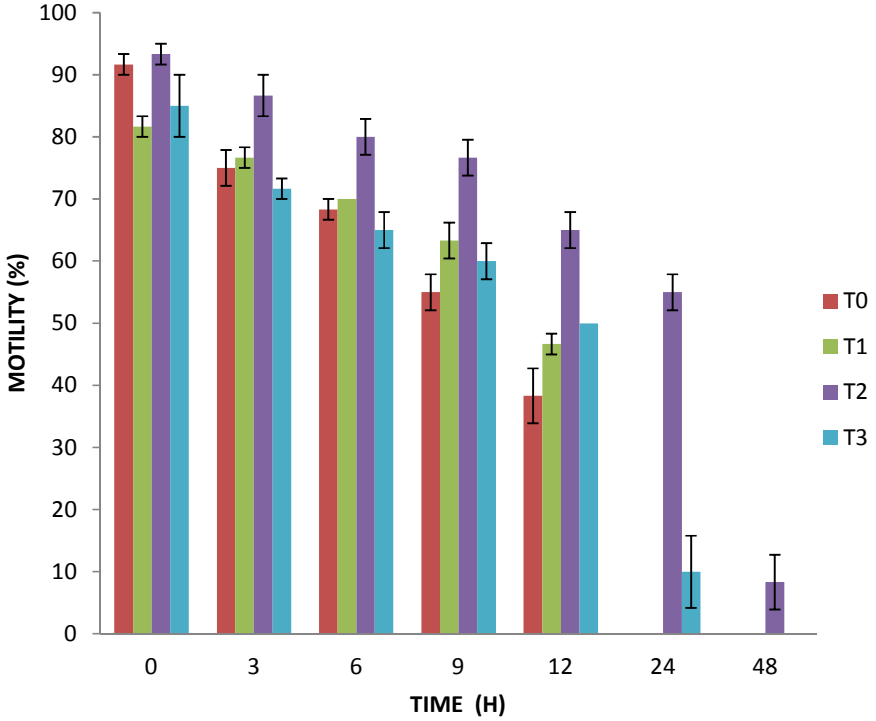

Fig. 1. Effect of vitamin C, E and their combination on the motility of chilled turkey Spermatozoa.

Key: T0 = Control, T1 = Vitamin C, T2 = Vitamin E, T3 = Vitamin C+E

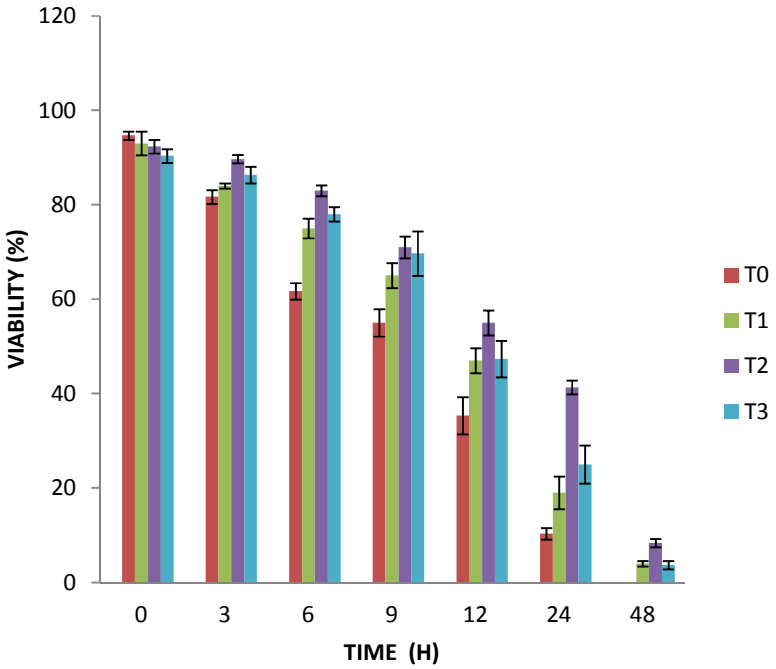

Fig. 2. Effects of Vitamins C, E and Their Combination on the Viability of Chilled Turkey Semen.

Key: T0 = Control, T1 = Vitamin C, T2 = Vitamin E, T3 = Vitamin C+E

observation when vitamin c was supplemented to semen diluents during *in vitro* studies. Surai [11] reported that vitamin C had no effect on the motility of human spermatozoa. Similar result was reported in rams [12] and horses [13]. Furthermore, vitamin C was reported to be a powerful antioxidant when peroxyl radicals are located in the aqueous phase but a poor scavenger for ROS within the lipid membrane [14]. [15] observed that vitamin C could function as an antioxidant at lower concentration and as a pro-oxidant at a higher concentration. At the concentration used in this study, vitamin C did not yield a better motility values over the control for 24 hours of storage. There was a more gradual reduction in semen motility in group T2 (VIT E) and T3 (VIT C+E) over a more prolonged period of storage. This finding was in agreement with the reports of [10] and [16]. The possible reason for the gradual decrease in semen motility for T2 and T1 may be attributed to their solubility and distribution of the antioxidants within and between the semen components.

Vitamin E has a larger distribution within the plasma membrane of spermatozoa and its lipid solubility allows it to suppress peroxidative damage across the plasma membrane. Vitamin E supplemented semen (T2) displayed a slower rate of mean motility decrease and was followed by T3 (VIT C+E). This result was in agreement with the reports of [10] and [17]. However [18] reported that the addition of vitamin E to cock semen did not show any difference in motility between the control and treated group when the semen was preserved for 24 hours in vitro. The combination of vitamin C and E showed slower motility decrease over the period of storage and this result could be attributed to the synergistic action between vitamin C and E as reported by [19] Although vitamin E was reported to inhibit ROS generation, the availability of vitamin C increases the antioxidant activity of vitamin E by regenerating the reduced vitamin E radical (alpha-tocopheroxyl radical) back to alpha tocopherol [20] for continuous antioxidative action or it can be stored [21].

4.1 Effect of Vitamin C and E on the Viability of Chilled Turkey Semen

The viability results from this study showed that antioxidant supplementation of turkey semen diluents improved the viability of stored turkey semen over the control, however, none of the antioxidants was able to protect against viability loss during the period of study. There was no difference in viability between the treatment groups and the control immediately after dilution. However, there was a progressive loss of viability across the treatment groups and control over the time period of the study. This report was in agreement with the report of [22] who reported an improvement in semen quality of roosters supplemented with vitamin C and E. The result from this study was also in agreement with the reports of [23] who reported an improvement in the viability of dog semen supplemented with ascorbic acid. The possible better viability result of vitamin C (T1) compared to the control could be attributed to the efficiency of ascorbic acid as an antioxidant which scavenges ROS thus protecting sperm membrane against lipid peroxidation. The result from this study showed that vitamin E (T2) had a better viability compared to vitamin C (T1), a combination (T3) and control (T0) over the storage period. The result from vitamin E supplementation from this study is supported by the earlier report of [10]. Similar results were also reported by [24] [18] who both observed that supplementing semen extender with vitamin E improved spermatozoa quality parameters including motility and viability of rooster semen. Similar reports were also documented in boars, rams, human and rats by [13, 16, 18], respectively. The maintenance of semen viability by addition of vitamin E to the semen diluents in this present study could be attributed to the high lipid solubility and membrane-stabilizing antioxidant effect of vitamin E within the cell membrane as proposed by [18].

The viability preservation result obtained from a combination of vitamin C and E (T3) in comparison to vitamin C (T1) and the control (T0) from this study was in agreement with the findings of [24] who observed a synergistic effect between Vitamin C and E and showed that the combination of vitamin E and C enhanced the activities of other antioxidant enzymes significantly. He also reported that vitamin C and E helped to reduced the risk of chronic diseases and cells damage related to oxidative stress. Several studies have shown that the association of ascorbic acid and their derivatives with vitamin E enhanced the antioxidant capability of both the two vitamins [25]. In studies with human

subjects, vitamin C supplementation increased plasma lipid standardized α vitamin E [26]. [10] reported that the addition of ascorbic acid did not improve maintenance of viability during chilled storage of turkey semen. [27] also reported that vitamin E supplementation did not improve turkey sperm motility and viability after 24 hours of cold storage.

5. CONCLUSION

It can be concluded that the supplementation of diluents of turkey semen with antioxidant Vitamin C and E improves the motility and viability of turkey semen during in-*vitro* storage.

ETHICAL APPROVAL

The study was approved by Ahmadu Bello University animal ethics committee to carry out the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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