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Evaluation of Vitamins C and E on Spermeogram and Malondialdehyde Concentration in Chilled Semen of Nigerian Indigenous Turkey Toms (*Meleagris gallopavo*)

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Authors' contributions

This work was carried out in collaboration among all authors. Author WOE designed the study. Author ROO performed the statistical analysis. Author JMM wrote the protocol and wrote the first draft of the manuscript. Authors ESI and IGOK managed the analyses of the study. Author IOS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The study aims to evaluate the effect of antioxidant vitamins C and E on spermeogram and malondialdehyde concentration in chilled semen of Nigerian indigenous turkey toms (*Meleagris gallopavo*). The results from this study showed that supplementation of the diluents of turkey semen with antioxidants resulted in improvement in spermatozoa motility, pH, volume and

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reduced rate of lipid peroxidation compared to the control (T0). Vitamin E recorded a better result in relation to motility, viability and lipid peroxidation rate better than a combination of vitamin C and E. Improvement in semen concentration of turkey tom were recorded in the semen of turkey extended with vitamin C. These results demonstrate that lipid peroxidation is a significant factor affecting the fertility of stored turkey sperm and those methods to prevent or reduce lipid peroxidation remain to be elucidated.

Keywords: Vitamin C; Vitamin E; semen; malondialdehyde; turkey toms.

1. INTRODUCTION

Lipid peroxidation of sperm membrane lipids is a major cause of decreased motility and fertilizing ability of mammalian spermatozoa [1]. Avian semen is characterized by a high levels of unsaturated fatty acids within their phospholipids [2]. This makes the semen of turkeys susceptible to lipid peroxidation and spermatozoa deterioration during storage [2]. Oxidative stress occurs when the production of potentially destructive reactive oxygen species (ROS) exceeds natural antioxidant defenses resulting in cellular damage [3]. Toxic lipid peroxides are reported to cause impairments of sperm cells and may play a major role in the etiology of infertility in males [4]. Malondialdehyde (MDA) is one of the final products of lipid peroxidation in seminal plasma [5]. It is an index of lipid peroxidation which may be a diagnostic tool for the analysis of infertility [5]. Previous studies have demonstrated a direct link between compromised poultry sperm function after *in-vitro* storage and lipid peroxidation [6]. Avian semen contains natural antioxidants including ascorbic acid, tocopherol, catalase, glutathione peroxidase and superoxide dismutase that help to balance lipid peroxidation and prevent excessive peroxide formation [7]. However, the endogenous antioxidant activity of seminal plasma and spermatozoa may not be enough to prevent peroxide damage after extension and *in-vitro* storage thus supplementation with antioxidants could improve the semen shelf life [8].

Suppression of lipid peroxidation through the addition of antioxidants vitamins C and E to the diluents has been achieved with relative success in chicken semen [8]. These antioxidants block the production of ROS or counteract oxygen toxicity. However, there is a paucity of information on the effect of different antioxidants and their combinations in preserving semen of turkeys. Also, previous studies on poultry semen in Nigeria were carried out using chickens; hence there is is a paucity of information on the effects of antioxidants on other poultry species like turkeys.

Despite the numerous advantages of artificial insemination over natural mating in turkeys, turkey semen cannot 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, this research is designed to evaluate the effect of Vitamins C and E on Spermeogram and Malondialdehyde Concentration in Chilled Semen of Nigerian Indigenous Turkey Toms (*Meleagris gallopavo*).

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. The mean daily temperature during season is 25 ^oC with a mean relative humidity of 72%. The dry season lasts from November to April, with mean daily temperature ranges of 14 – 36 ^oC and relative humidity of 20 – 30% [9].

2.2 Experimental Turkeys

Twenty (20) healthy indigenous turkey toms 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 helminths and blood parasites before 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.

2.3 Housing and Management of the Turkeys

Turkey toms were housed individually in cages and allowed to acclimatize for 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 analyzed at the Animal Nutrition Laboratory Department of Animal Science, A.B.U, Zaria. Water was provided *ad-libitum.*

2.4 Initial Semen Collection and evaluation

Semen was collected from the toms by abdominal massage technique as described by [10] 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 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 (Fig. 1). The semen was collected into a calibrated semen vials and evaluated for volume, color, pH, concentration, motility (gross and individual), live-dead ratio and morphology.

2.5 Semen Evaluation

Collected semen samples were evaluated as described by [11] for:

- Volume**:** The volume was be measured immediately after collection using a calibrated tube (Fig.2).
- 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 hemocytometer as described by [11]. The semen concentration was calculated as follows:
  Concentration (sperm cells/ml) = Average count x dilution factor $x10^4$
- Live spermatozoa percent**:** was determined as described by [12]. A drop of semen sample was placed on the edge of a clean grease free glass slide and three drops of eosine-nigrosine 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 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 were determined by making a thin smear from the mixture of semen sample and eosine-nigrosine on clean grease free glass slide. one hundred sperm cells were counted per slide using light microscope at x 40 magnification [12].

2.6 Malondialdehyde Assay

Malondialdehyde was measured by the Thio Butyric Acid Reactive Substances (TBARS) method as described by [13]. A detection kit was purchased from Radox (Radox laboratory Ltd, United Kingdom). 0.1µl of semen was mixed with 0.1µl of TBA reagent (15% trichloro acetic acid + 0.25NHCL). A coloured product was formed. The optical density was measured at 450 nm using a spectrophotometer (Rayto, Japan) and the MDA concentration was measured in µmoles/ml.

2.7 Diluted Semen Evaluation

2.7.1 Diluent preparation

Egg yolk citrate (EYC) diluent was prepared at ambient temperature as described by [13] 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) were added to the mixture. The contents were shaken vigorously to ensure proper mixing (Figs 3, 4 and 5).

2.7.2 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^{o}C for storage (Fig.8).

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

Fig. 1. Semen collection

Fig. 2. Measurement of semen volume

2.8 Data Statistical Analyses

Data collected from this study was expressed as 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.

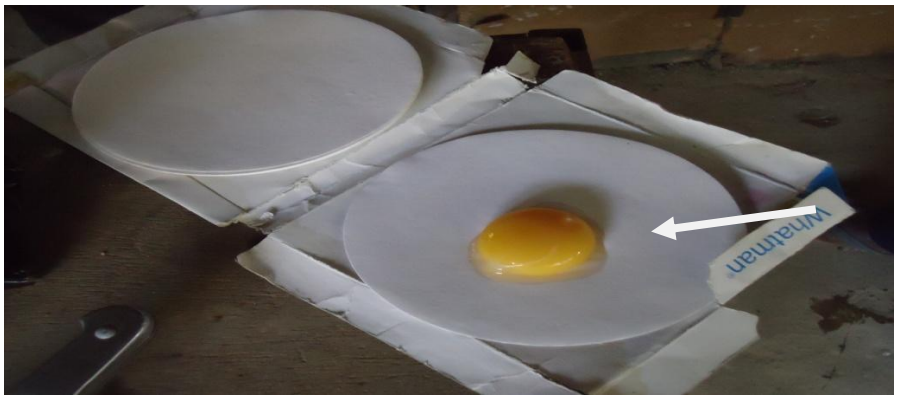

Fig. 3. Egg yolk placed on a filter paper

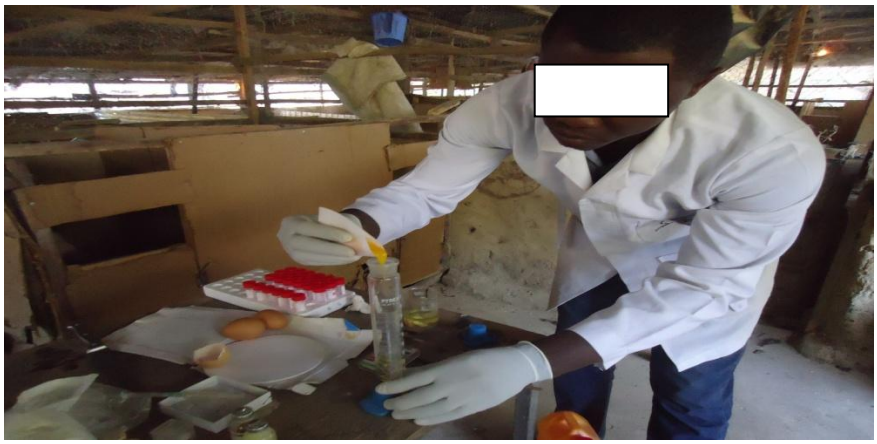

Fig. 4. Diluent Preparation

Fig. 5. Diluent Preparation

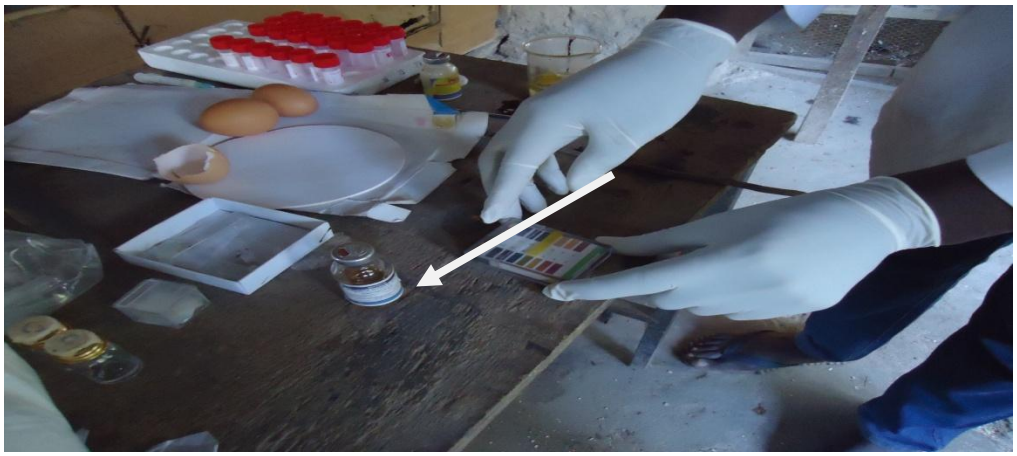

Fig. 6. Reading pH of diluent

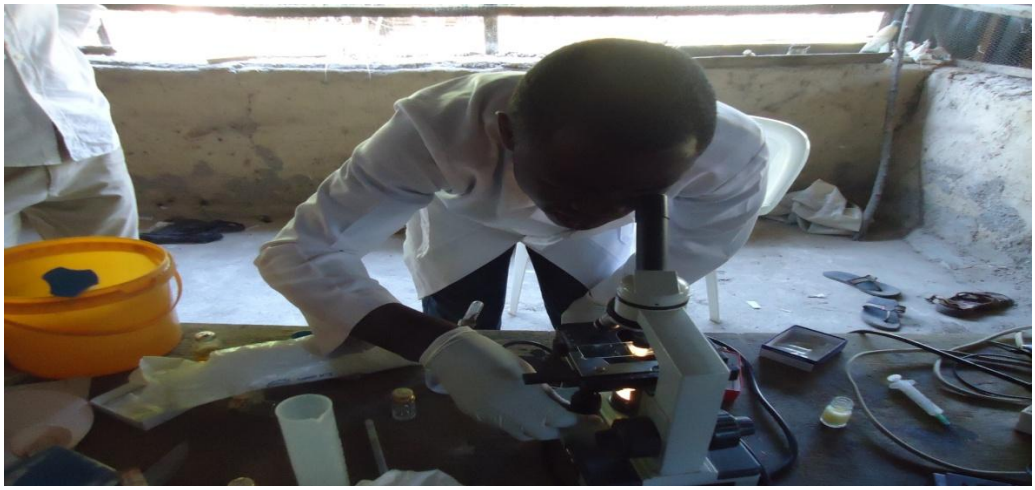

Fig. 7. Microscopic examination of collected semen

Fig. 8. Packaging of Diluted semen into biju bottles

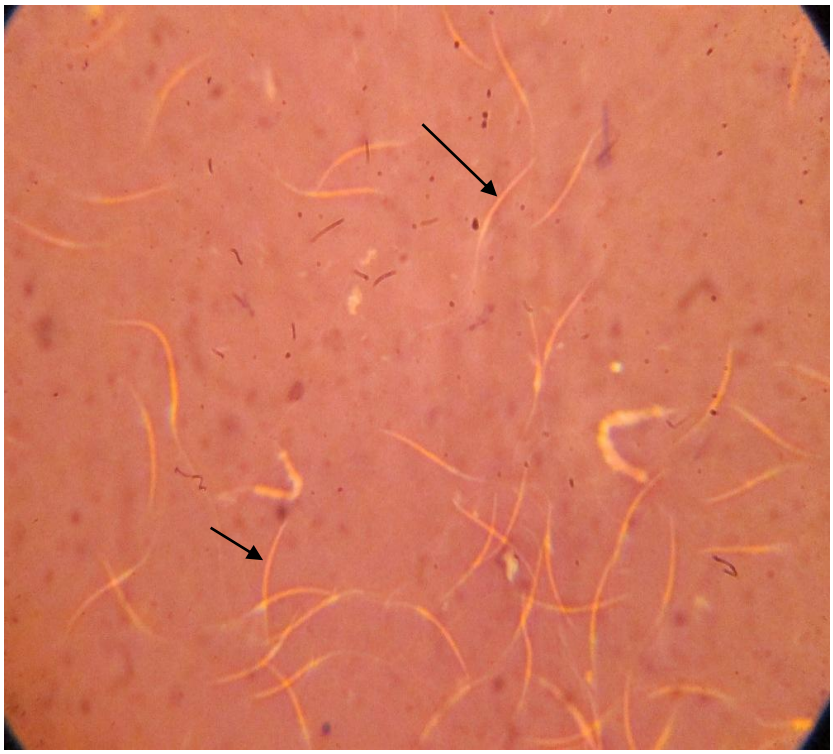

Fig. 9. A photomicrograph of eosin-nigrosin stained slide showing live spermatozoa. (Black arrows)

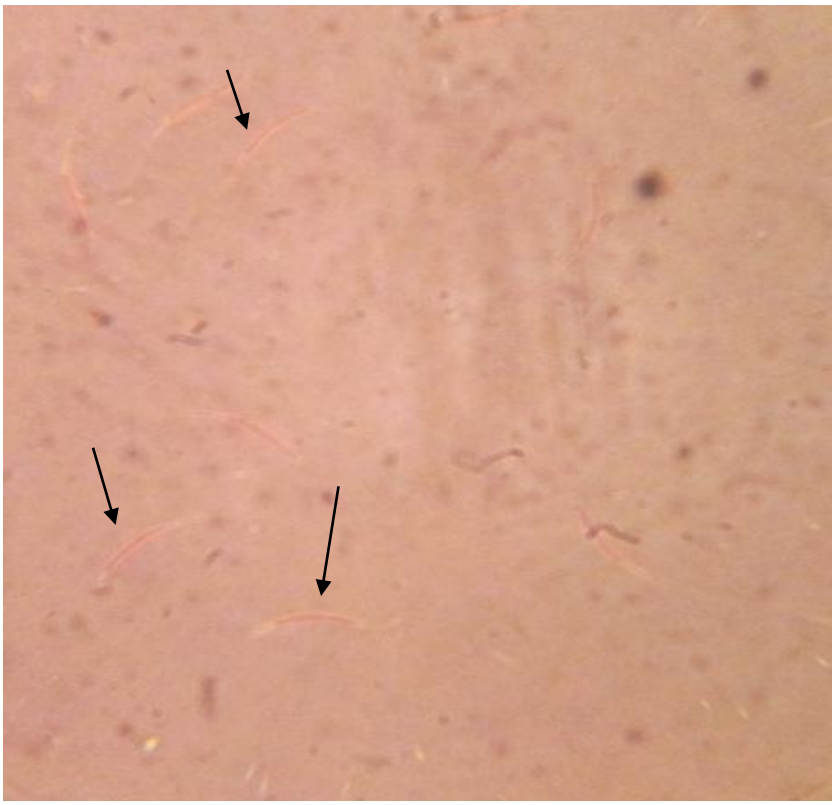

Fig. 10. A photomicrograph of eosin-nigrosin stained slide showing dead spermatozoa. (Black arrows)

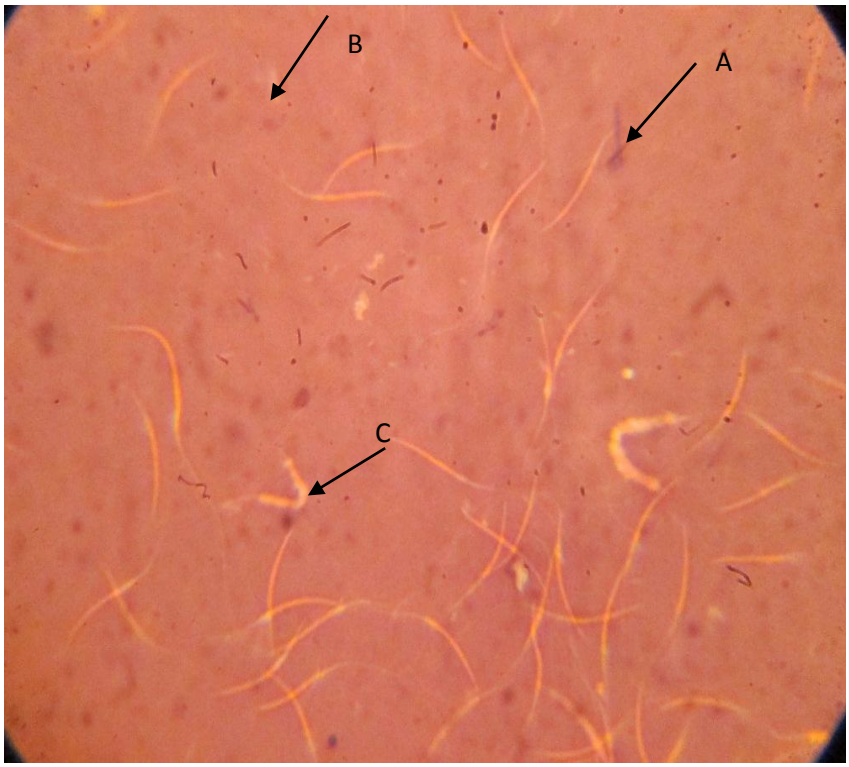

Fig. 11. A photomicrograph showing some spermatozoa abnormalities; A-Coiled tail, B- Loose head and C- Bent mid-piece

3. RESULTS AND DISCUSSION

The effect of vitamins C and E on semen morphometry and MDA activity are shown in Table 1. There were no significant differences ($p>0.05$) across the treatments for volume, colour, concentration, pH, percent live, percent deformed and MDA activity. The means ± SEM of semen volume range was 0.17± 0.01-0.24±0.02 ml. The colour was averagely creamy to milky 2.43±0.10. The means of spermatozoa motility, pH, concentration, percent live, percent deformed and MDA in T3 group were 91.75±0.97%, 6.94±0.10, $6.80x10^9$±0.05 sperm/ml, 91.75±0.90%, 8.15±0.59% and 0.74±0.03 µl/ml respectively.

The mean semen volume range of indigenous turkey toms (0.17-0.24 ml) was lower than the acceptable range limit of 0.25-0.35 ml and 0.26-0.35 ml for toms reported by [14]. The overall mean volume from this study was lower than 0.5 ml in cocks reported by [14] and also below the acceptable range of 0.25-2 ml presented by [14] for chickens. Variation in ejaculate volume could be attributed to breed difference, season, age, frequency of semen collection and level of nutrition [15].

Table 1. Effect of antioxidants vitamin C and E supplementation on semen morphometry and MDA activity of turkey toms

Traits	T0	T1	T2	T3
Volume (ml)	0.24±0.02	0.17±0.01	0.18±0.01	0.18±0.02
Colour	2.74±0.10	2.15±0.10	2.50±0.11	2.35±0.10
Motility (%)	89.75±1.05	89.50±1.02	91.00±1.00	91.75±0.97
Conc (x 10^9/ml)	6.90±0.07	6.97±0.02	6.85±0.08	6.80±0.05
pH	6.71±0.01	6.83±0.10	6.92±0.02	6.94±0.10
% Live	86.30±1.61	92.65±1.06	91.47±0.80	91.75±0.90
% Deformed	7.15±0.63	7.05±0.66	8.75±0.74	8.15±0.59
MDA (µl/ml)	0.72±0.03	0.73±0.03	0.72±0.03	0.74±0.03

T1= antioxidant vitamins C (4 mg/ml), T2= antioxidant vitamins E (8 mg/ml), T3= antioxidant vitamins C+E (4 mg + 8 mg/ml), No antioxidant was added to the extender in the control group (T0).

The semen colour of indigenous turkey toms in the current (milky-creamy) correlates with reports of [16] who attributed any deviation in the colour of poultry semen from milky to creamy colour to contaminants. The mean spermatozoa concentration in this study 6.66 ± 0.66 x 10^9 was higher than values reported for indigenous turkey toms by [16] and was lower than the value reported for exotic turkey flocks by [16]. The variation in sperm concentration could be influenced by breed, nutrition, season and method of semen collection [14].

Motility, expressed as the percentage of spermatozoa moving through the power of their tails, is used as an indicator of spermatozoa viability. The mean motility value (90.50 ± 1.01) in the present work was within the established normal range proposed by [16].

The pH of indigenous turkey semen obtained from our current findings (6.85± 0.05) was within the range previously reported for poultry by [17]. A lower semen pH can be attributed to contamination mostly by urine during collection [17]. The percent live spermatozoa is used as an index of viability measurement. In the present work, the live sperm percent was (90.54 ± 1.09%). This value was slightly higher than the value reported by [18] where it was 86.20% in fresh semen of exotic turkey toms. The current percent mean deformed spermatozoa was (7.77 ± 0.65%) which was lower than the value (14.36 ± 1.17%) reported for indigenous toms by [14].

Concerning the MDA concentration, the present study revealed that the MDA concentration in fresh semen samples from indigenous turkey toms was similar with the reports of [19] which demonstrated that some lipid peroxidation naturally occurs within the male reproductive tract and this level of lipid peroxidation does not impair fertility in turkeys. The our findings related to the MDA concentration were in agreement with the findings of [19] which demonstrated that lipid peroxidation differs in individual male birds.

4. CONCLUSION

Our data concluded that the synergy between antioxidant vitamin C and E alone was not sufficient to deter lipid peroxidation during storage of turkey semen. Antioxidant supplementation of turkey semen extenders could not prevent lipid peroxidation during in vitro semen storage at refrigeration temperature. Semen not intended for immediate use in AI should be extended and supplemented with vitamin C and E and such extended semen should not be preserved longer than 24 hours before use.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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