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Comparative effects of two naturally formulated extenders on tom semen preserved for 72 hours at 4 ^{0}C

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Abstract

Tom semen dilution and liquid storage requires the identification of suitable extenders in order to expedite genetic improvement and transmission of turkey germplasm. The purpose of this study was to evaluate the ability of two naturally made semen extenders, tris egg-yolk orange (TEYO) juice and tris coconut-water orange (TCWO) juice, to preserve semen. For the experiment, five mature toms at the age of reproduction were utilized. Each tom was ejaculated individually followed by pooling of semen. The pooled semen was added to the extender in the ratio of 1:3 (semen: extender). Experimental design was completely randomized design. Samples were immediately examined for microscopic semen quality and further kept for a period of 72h. The microscopic semen quality factors examined were acrosome integrity, viability membrane, and motility in freshly extended semen and semen stored for 72h at every 12h interval. The results showed that percentage sperm motility was significantly ($p < 0.05$) different among the treatments. Percentage live sperm of TEYO and TCWO extended tom semen were significantly different ($p < 0.05$) compared to the neat semen from 12 to 72h of storage. Significantly greater ($p > 0.05$) sperm membrane integrity was recorded for TCWO extended tom semen compared to TEYO extended and neat tom semen from 12 to 72h of storage. In conclusion, TCWO extender preserved tom sperm for longer period of storage compare to TEYO extender and un-extended semen, which was evident in motility and membrane integrity result.

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1. Introduction

Since artificial insemination enables a greater utilization of genetically superior animals with high output performance, it has been regarded as a desirable approach in the chicken business (Blanco and Hofle 2004). A significant number of females have been rapidly exposed to genetic material from a limited number of exceptional sires by the introduction of Assisted Reproductive Biotechnology (ART). Artificial Insemination (AI) is the biotechnology most frequently utilized in cattle farms across developed and developing nations (Alvarino 2000). Nonetheless, the practice is primarily restricted to research stations, universities, and a few sizable commercial livestock farms in Nigeria. Numerous advantages of this strategy include genetic selection, cycle-based production, longer fertility even in adverse seasons of the year, and more effective breeding programs (Carluccio et al. 2004).

However, during AI procedures, semen quality and fertility rates are typically impacted, particularly when semen is held for a short or extended period of time (4–72 hours) prior to use. Lipid peroxidation-induced seminal oxidative stress is one of the main causes of low-quality semen during in-vitro preservation (Henkel 2011; Balogun et al. 2015). Furthermore, a high concentration of polyunsaturated fatty acids (PUFAs) during storage raises a cell's vulnerability to lipid peroxidation and free radical attack (Balogun et al. 2016). Despite the fact that sperm cells often contain antioxidants to protect them against reactive oxygen species, it appears that this defense mechanism is insufficient (Saleh et al. 2002). Because of this, sperm cells need a medium that provides them with enough energy, nutrients, and antioxidants to sustain their activities from ejaculation to fertilization.

Diluents used in semen extension should have the special capacity to shield spermatozoa from oxidative damage. Antioxidant-supplemented semen extender may lessen the effects of reactive oxygen species brought on by lipid peroxidation. It has been demonstrated that adding antioxidants to the extender increases the vitality of the sperm in bovine semen (Krzyzosiak et al. 2000). The majority of studies conducted nowadays concentrate on natural extracts and infusions that are utilized in semen extenders to preserve animal sperm (Sansone et al. 2000).

Additionally, it has recently been demonstrated that adding fruit juices from *Citrus sinensis* (orange), *Cucumis sativus* (cucumber), *Ananas comosus* (pineapple), and other fruits to

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semen extenders improves the quality of spermatozoa under storage (Daramola et al. 2016).

Coconut water has been found to be an appropriate natural component of extender for farm rooster and cock semen (Balogun 2019; Balogun 2021). It is also characterized by its high contents of antioxidants as expressed by the phytohormones (Tezcan et al. 2005), sugar, vitamins, electrolytes, and amino acids (Yong et al. 2009). According to Nascimento et al. (2009), coconut water can be used to cultivate and mature ovine, caprine, and bovine oocytes and embryos as well as to capitate sperm in pigs *in-vitro*.

Additionally, it has been demonstrated that egg yolks are a strong option for protecting sperm from cold shock and are high in tocopherol, a precursor to vitamin E. In contrast to single pure active fractions, scientists are becoming more interested in the possible health advantages of phytochemicals and the synergistic effects of their numerous constituents (El-Sheshtawyet al. 2017). Due to their effectiveness in preventing oxidative damage, fruit juice's natural antioxidants are becoming more and more preferred for their biological and protective properties when used as a component of semen extenders (Balogun 2019). Thus, the purpose of this experiment was to study the comparative effects of the orange juice and the coconut water based extenders on the diluted tom semen at different storage time periods.

2. Materials and methods

2.1 Tris egg-yolk orange juice extender (TEYO) preparation

Fresh quail eggs were gathered from the farm. Methylated spirit was used to sterilize the egg shells before breaking off the pointed tip to extract the yolk. Filter paper was used to help separate the yolks from the albumen. The yolks were gathered into a beaker and given a good shake. A pH 7.2 Tris buffer was made using tris salt and double-distilled water. 75% tris buffer was combined with 25% egg yolk, and the mixture was thoroughly mixed. Furthermore, orange juice was incorporated into the blend at the rate of 10%. The extender was kept in a refrigerator at 4°C until it was needed.

2.2 Tris coconut-water orange juice extender (TCWO) preparation

The mature coconuts were procured from the market. The cap was punctured in order to release the water into the beaker. Filter paper was inserted into a funnel to filter the water. A pH 7.2 Tris buffer was made using double-distilled water. 50% coconut water by volume was added to the tris buffer and thoroughly mixed. Lastly, the mixture was mixed with 10% orange juice. The extender was kept in a refrigerator at 4°C until it was needed.

2.3 Tom management and training for semen collection

For the experiment, five fully grown males between the ages of 30 and 40 weeks were utilized. They were housed in a pen together. Turkey breeder feeding criteria were followed when providing feed and water: each hen received 180 g of feed each day, while each tom received 220 g. By employing a modified method of Balogun et al. (2015) for semen collection in poultry, the toms were trained for two weeks prior to collection of semen. This involved milking the semen immediately by pushing the fleshy area of the tom cloaca and simultaneously employing the mid-stroke and abdominal massage technique. Typically, semen was taken once a week for four weeks to have adequate sperm reserves.

2.4 Experimental design

In order to prevent bias resulting from variations in the quality of each tom semen, ejaculates from all toms were gathered and combined. The semen was divided into three portions, two portions of pooled semen were added to extenders (one each to TEYO & TCWO) in 1:3 ratio (semen: extender) while the last portion was void of extender. The samples were kept for seventy-two hours at 4°C. Complete randomization was the method employed in the experiment. There were three treatments (Un-extended semen, TEYO & TCWO extended semen) in the experiment, and three trials were run. The microscopic semen characteristics such as motility, viability, membrane integrity, and acrosome integrity were measured and recorded both freshly undiluted and diluted semen and at every 12 hour interval.

2.5 Semen evaluation

Progressive motility

10 µl of both diluted and un-diluted semen samples were put on a slide that had been warmed, covered with a cover slip, and examined at 400X magnification to identify the progressive movement of the motile sperms.

Sperm livability

Eosin-nigrosin stain was prepared in order to evaluate the viability of sperm. 10 µl of semen was placed on a stage warmer, and after applying two drops of eosin-nigrosin stain with a dropper, it was left for two minutes. A clean, pre-warmed glass slide was used to create a thin smear from the semen stain mixture. After the slide had air dried, the stained slide was evaluated using a bright-field microscope at 1000 X magnification. The livability proportion was calculated after about 200 sperm were counted and recorded. Sperm that were unstained/ partly stained and stained were regarded as live and dead, respectively. The percent viability was calculated by the formula:

$$\textit{Sperm liability (\%)} = \textit{No. of live sperms} / \textit{Total sperms)} \times 100$$

Membrane integrity

The application of the hypo-osmotic swelling test (HOST) method assessed the integrity of the sperm membrane. After the solution was ready, 200 µl of hypo-osmotic solution and 10 µl of semen were combined, and the mixture was incubated for 30 minutes at 37 °C. A sample drop was inspected for curled and uncurled tail spermatozoa at 400X magnification using a bright-field microscope. For every sample, the curled and uncurled characteristics were noted in about 200 sperms and percentage of spermatozoa with curled tail was calculated.

Acrosome integrity

The spermatozoa's acrosome integrity was evaluated using Giemsa stain (Watson 1975). A 10 µl diluted and undiluted semen samples smear were placed on sterile glass slides, allowed to air dry, and then fixed for 30 minutes in a 2% glutaraldehyde solution. The fixed slides were allowed to air dry for thirty minutes, and the smear was then incubated in Giemsa working solution for two hours. The slides were taken out of the stain, rapidly rinsed in double distilled water, allowed to air dry, and then viewed using a bright field microscope with oil immersion (1000X magnification). From each slide, at least 200 spermatozoa with intact and partially or fully injured acrosomes were counted in separate fields. The % acrosome integrity was determined as follows:

Acrosome integrity (%) = (No. of sperms with intact acrosome / Total sperms) x 100

2.6 Statistical analysis

Using SPSS 22 software, the data from each trial were combined and a one-way analysis of variance (ANOVA) was used to assess the effects of extenders on storage of sperm. The Ducan multiple range test was used to separate the means.

3. Results

Percentage sperm motility of tom semen preserved with TEYO and TCWO extenders is presented in Table 1. Percentage sperm motility was significantly higher in TCWO at all the periods of storage compared to neat semen and TEYO extended semen ($p < 0.05$). Percentage sperm motility in TCWO and TEYO extended semen remained above average (50%) for 48 hours and 36 hours of storage, respectively. However, motility percentage of neat semen decreased below average within just 12 hours of storage. Percentage live sperm count of tom semen preserved with TEYO and TCWO extenders is presented in Table 2. Percentage live sperm count of TEYO and TCWO extended tom semen were significantly higher ($p < 0.05$) compared to the values obtained for neat semen from 12 to 72h of storage. However, percentage live sperm count above 60.0% were recorded for both un-extended and extended tom semen throughout the storage period.

Percentage sperm membrane integrity of tom semen preserved with TEYO and TCWO extenders is presented in Table 3. Significantly higher ($p < 0.05$) sperm membrane integrity was recorded for TCWO extended tom semen compared to TEYO extended and neat tom semen from 12 to 72h of storage, except at 12h storage where TEYO extended tom semen was statistically similar ($p > 0.05$) to TCWO extended tom semen. The tom semen extended in both TEYO and TCWO extenders had sperm membrane integrity of 53.81% and 57.42% at 36h and 48h storage, respectively, which was above average compare to un-extended tom semen that has percentage sperm membrane integrity below 50.0% starting from 24h of storage.

Percentage sperm acrosome integrity of tom semen preserved with TEYO and TCWO extenders is presented in Table 4. No significant ($p > 0.05$) differences was observed in percentage sperm acrosome integrity throughout the period of storage. 100.0% intact acrosome was recorded for both the extended and neat semen throughout the storage periods except at 12h and 72h where TEYO extended tom semen had 99.17 % percentage sperm acrosome integrity values.

Table 1 Comparative effects of TEYO and TCWO extenders on tom sperm motility at different storage periods

Extender	0 h	12 h	24 h	36 h	48 h	60 h	72 h
Neat semen	89.17	54.17^{b}	36.67^{c}	11.67^{c}	7.50^{c}	2.50^{b}	0.00^{b}
TEYO	88.33	82.50^{a}	60.00^{b}	50.00^{b}	38.33^{b}	5.83^{b}	5.83^{ab}
TCWO	89.17	84.17^{a}	74.17^{a}	61.67^{a}	56.67^{a}	22.50^{a}	10.00^{a}
SEM	1.04	3.70	4.16	5.42	5.20	2.79	1.92

Means with different superscript letters a b c within the column differ significantly ($p < 0.05$)

TCWO: Tris coconut-water orange juice extender; TEYO: Tris egg-yolk orange juice extender; SEM: Standard error of Mean

Table 2 Comparative effects of TEYO and TCWO extenders on tom sperm live count at different storage periods

Extender	0 h	12 h	24 h	36 h	48 h	60 h	72 h
Neat semen	97.33	91.10^{b}	87.20^{b}	86.30^{b}	84.35^{b}	73.17^{b}	65.84^{b}
TEYO	97.00	94.94^{a}	93.25^{a}	90.83^{a}	89.17^{a}	86.00^{a}	78.83^{a}
TCWO	95.93	94.59^{a}	92.17^{a}	91.33^{a}	90.17^{a}	89.08^{a}	85.67^{a}
SEM	0.61	0.75	0.95	0.77	0.79	1.86	2.42

Means with different superscript letters a b c within the column differ significantly ($p < 0.05$)

TCWO: Tris coconut-water orange juice extender; TEYO: Tris egg-yolk orange juice extender; SEM: Standard error of Mean

Table 3 Comparative effects of TEYO and TCWO extenders on tom sperm membrane integrity at different storage periods

Extender	0 h	12 h	24 h	36 h	48 h	60 h	72 h
Neat semen	89.70	60.00^{b}	34.67^{c}	14.55^{c}	10.82^{c}	6.02^{b}	2.33
TEYO	88.80	82.80^{a}	57.67^{b}	53.81^{b}	39.76^{b}	13.4^{b}	8.40
TCWO	92.34	86.00^{a}	74.80^{a}	66.67^{a}	57.42^{a}	27.18^{a}	10.17
SEM	1.06	3.5	4.45	5.60	5.03	2.94	0.28

Means with different superscript letters a b c within the column differ significantly ($p < 0.05$)

TCWO: Tris coconut-water orange juice extender; TEYO: Tris egg-yolk orange juice extender; SEM: Standard error of Mean

Table 4 Comparative effects of TEYO and TCWO extenders on tom sperm acrosome integrity at different storage periods

Extender	0 h	12 h	24 h	36 h	48 h	60 h	72 h
Neat semen	100.00	100.00	100.00	100.00	100.00	100.00	100.00
TEYO	100.00	99.17	100.00	100.00	100.00	100.00	99.17
TCWO	100.00	100.00	100.00	100.00	100.00	100.00	100.00
SEM	0.00	0.28	0.00	0.00	0.00	0.00	0.28

TCWO: Tris coconut-water orange juice extender; TEYO: Tris egg-yolk orange juice extender; SEM: Standard error of Mean

4. Discussion

The results of this study clearly revealed that TCWO extender is a better extender for turkey semen dilution and preservation compared to TEYO extender. Notably, the survival of sperm cells in TCWO extender for 48 hours compared to TEYO extender appears unique especially under cold storage conditions. Its ability to maintain a steady sustenance of sperm motility, membrane integrity, and sperm livability above average for 48h period of preservation is probably because of the cytokinin and other chemical components like sugar, vitamin, minerals and amino-acid in coconut which are known to regulate metabolic activities of sperms (Jean et al. 2009). The results obtained corroborate the report of Taner and Ergum (2010) that coconut-based extenders is a better sperm preservative than Ringer's solution.

One of the factors that might have led to the rapid decline of the sperm motility in diluted samples after 36hr storage may be lack of sufficient available nutrients, energy, antioxidants for the *in-vitro* sperm survival and non-stable temperature of the liquid storage condition. This probably allowed quick depletion of metabolizable substrate by the competing microbes and reacting oxygen species (ROS) which practically compete with sperm cells for available nutrients in the media used for preservation. Similarly, the results of this study agree with the report of Norman (1962) that coconut extender encourages better semen quality. The lower motility observed in sperm stored in TEYO extender than sperm stored in TCWO extender throughout the storage periods conforms to the report of Taner and Ergum (2010).

The survival of higher sperm percentage above average for 48h in TCWO extender observed in the present study could be linked to essential constituents such as sugar, minerals and amino acids, and ions in coconut water (Vigliar et al. 1996) and antioxidant activities (Silva and Bamunuarachi 2009). The presence of antioxidants, sugar, vitamins, electrolytes and amino acids and essential inorganic compounds in the coconut extenders has been extensively reported in previous studies (Yong et al. 2009). The TCWO extender used in this study is considered to be an important buffer and nontoxic that determine the success of sperm storage. Analysis percentage of live spermatozoa showed no significant differences between extended and un-extended semen. The mean live sperm percentage is higher than the mean motile spermatozoa percentage (Bearden and Fuquay 1997) because the live sperms are not necessarily motile, some spermatozoa are not motile and still alive (Campbell et al. 2003).

5. Conclusions

In conclusion, TCWO extender preserved tom sperm for longer period of storage compare to TEYO extender and un-extended semen, and this was evident in motility and membrane integrity result.

Declarations

Funding: None

Conflict of interest: The authors declare that they have no conflict of interest arising out of this study

Ethical approval: Not applicable

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