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Optimizing Dilution Rate of Cock Semen with Natural Based Extender for Optimum Fertility

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ABSTRACT

Optimizing the dilution rate of poultry semen for artificial insemination use is a precursor to better fertility and effective use of outstanding sires. An experiment was conducted to standardize the dilution rate of cock semen with two formulated natural-based poultry semen extenders for optimum fertility. A total of 5 cocks and 25 hens of 45 weeks of age were used for the experiment. The experiment was in two phases (*in-vitro* and *in-vivo*) comprised of three different trials and lasted for 8 weeks. Extenders were constituted and semen was ejaculated from each cock and pooled. Pooled semen was divided into four portions and diluted with Tris egg-yolk orange juice extender (TEYOE) viz; (Neat Semen, 1:2, 1:3 and 1:4). Diluted semen was evaluated for motility, viability, membrane, and acrosome integrity. This procedure was repeated on three different days consecutively. The same process was done with Tris coconut water orange juice extender (TCWOE). The best two dilution rate for each extender was selected and further use for artificial insemination of hen for fertility trials. Fertilizing ability results of each extender at 1:2 and 1:3 dilution rate was compared. Microscopic semen quality result revealed that 1:2 and 1:3 dilution rates were best for cock semen extension for both extenders as most semen quality parameters like motility, viability, membrane, and acrosome integrity had higher value of above 90%. However, fertilizing ability revealed that 1:3 dilution had higher values compared to 1:2 in both extenders. Conclusively, only TCWOE 1:3 dilution had a better higher comparable value to neat semen.

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1.0 Introduction

Artificial Insemination practice is increasingly popular in other poultry species other than turkey. In the last few decades, more farms in Nigeria now rely on artificial insemination (A.I) for their broiler breeders. For instance, broiler breeder's fertility decline continuously as more males are selected for growth. Artificial insemination (AI) is a biotechnological tool used to improve poultry and livestock productivity and yield. 80–85% fertility is usually achieved with natural mating. However, fertility can be further increased by 5–10% simply through augmentation with A.I to the breeding program (Gee *et al.,* 2004).
Complementing A.I advantages, are the use of extension and preservation of semen, mostly from outstanding sires. Dilution of neat semen is more advantageous, as more hens can be inseminated from a single ejaculated semen sample. Semen dilution with an extender for poultry is paramount because cock semen is very small in volume and highly concentrated with sperm cells, containing approximately 5000 $\times 10^6$ sperm/ml Gordon (2005). However, the preference of semen extender is also a relevant aspect of semen processing for A.I (Peterson *et al*., 2007).

The majority of diluents used are salt solutions. Mostly, these solutions cater for an osmotic pressure of 300-400 m Osm and a pH of 7.0-7.4 for the poultry semen. However, they are effective for preserving semen in a short duration (Miškeje *et al*., 2103). But, no standard effective extender for poultry semen yet as in cattle, although a variety of studies have been conducted by different scientist till present which includes the formulation of the extender with various salt components, dilution rate, insemination period, vaginal depth, and frequency of AI, a number of spermatozoa inseminated and fertility duration analyzed but discerning the benefits of different diluents is difficult.
Glutamic acid is said to be the most important chemical component of poultry seminal plasma and is known as a standard component of diluters (Siudzinska and Lukaszewicz, 2008). Egg-yolk and coconut-water known to contain a considerable amount of glutamic acid, vitamin E and major electrolyte, an evident for their antioxidant potentials have been a major component of cattle and some other farm animal species diluents. Egg yolk is generally accepted to be an effective agent in semen extenders for protection of spermatozoa against cold shock and the lipid phase transition effect (Aboagla and Terada, 2004).
Moreover, optimizing the potentials of egg-yolk and coconut-water by supplementing it with orange juice as a conjugate antioxidant source has been reported to enhance semen quality *in-vitro* and *in-vivo* and subsequently egg fertility percentage (Balogun, 2019).

In-addition, Penfold *et al.* (2001) reported the advantages of diluting freshly collected cock semen at 1:1dilution rate with bestvile poultry semen extender (BPSE) on mass motility and progressive motility status regardless of the temperature (24^{o}C at 4^{o}C) while further dilution in BPSE (1:10 or 1:100) resulted in a transient increase and rapid losses in progressive motility ability of the sperm cells. Hence, maintaining the semen quality of poultry sperm *in vitro* shortly before and during insemination has been observed to require suspending it in a solution capable of preserving its fertilizing ability till the sperm is inseminated and probably inside the hen oviduct. Thus, identifying the proper and optimum dilution ratio is paramount and may be an important step in achieving better-diluted semen quality and subsequently optimum fertility.

2.0 Materials and Methods

The present studies were planned to investigate the possibility of standardizing and ascertaining the optimum dilution rate of rooster semen extended with tris egg-yolk orange juice (TEYO) and tris coconut-water orange juice (TCWO) extenders. The studies include microscopic semen quality and fertility assessment of freshly diluted semen.

2.1 Preparation of Fruit Juices:

Ripe oranges were purchased from the market. The juice was extracted from orange with a juice extractor and was stored at -20^o C inside the refrigerator till use.

2.1.1 Preparation of extenders

2.1.1.1 Preparation of Tris egg-yolk orange juice extender

Preparation of tris buffer was done by adding 3.785g of Tris to distilled water and the pH was adjusted to 7.4. Two domestic chicken eggs weighing approximately 45-50g were freshly collected daily from the poultry farm. Each egg was gently broken with the use of forceps, separation of albumen from yolk was gently done by avoiding damage of the yolk. The yolk was collected on the filter paper, to get rid of the albumen, and further removed the yolk from the membrane covering it by puncturing with the use of small tips. Tris-egg yolk (TEY) extender was prepared by mixing egg 20% yolk in the Tris buffer. 10% of orange juice was further supplemented in TEY extender.

2.1.1.2 Preparation of Tris coconut-water orange juice extender

Preparation of tris buffer was done by adding 3.785g of Tris to distilled water and the pH was adjusted to 7.4. Coconut fruits were procured from the market, the shell was broken and water was drained from it into a beaker, it was decanted and filtered. Coconut-water was kept at -20^{o} C inside refrigerator for further use. For every trial, Coconut-water was thawed and added at the ratio of 1:1 to tris buffer. 10% orange juice was finally supplemented in tris coconut-water extender.

2.2 Experimental Animals

5 marshal breeder roosters of 45 weeks of age were used in this study. The roosters were subjected to semen collection training for a period of two weeks. The roosters were caged in individual cells at the poultry unit of Obasanjo farms limited. Feed and water were offered according to the breeder recommendations. Semen was collected twice a week with the modified poultry semen collection protocols of Balogun *et al.*, 2015, ejaculates from each cock were collected into a glass funnel and pooled per trial. Feaces/urine/Bloodstained contaminated semen was discarded and was examined for individual progressive motility. The extender and semen were kept at 37^{o} C and where processed for dilution within 5-10 mins.

2.3 Standardization of dilution rate Tris Egg-Yolk and Coconut-water orange juice extender

Ejaculated Pooled semen was divided into 4 equal parts consisting of four treatments viz: (Neat Semen), Extended semen (1:2, 1:3 and 1:4). The extended and unextended semen samples were evaluated at the storage periods interval for motility, viability, membrane, and acrosome integrity. This was done separately for each extender.

2.4 Experimental Design

The experimental design used in the standardization of rooster semen dilution ratio was a completely randomized design with the use of the following linear model:

$$X_{ijk} = \mu + A_i + e_{ijk}$$

where

A_i = Effects of dilution ratio on the i^{th} group

e_{ijk} = Random error ~NIE (0, σ_e^2)

All data collected were analyzed using SPSS software statistical analysis package.

2.5 Analysis of Semen

2.5.1 Progressive motility

5ul of un-extended and extended semen samples were placed on a pre-warmed slide, covered with a coverslip, and observed under a light microscope at 400X.

2.5.2 Sperm livability

Sperm livability was assessed by preparing Eosin-nigrosine stain. From the semen stain mixture, a thin smear was prepared using a clean, grease-free pre-warmed glass slide and the stained slide was examined under oil immersion (1000 X) using a bright-field microscope after the slide is air-dried. Stained / partially stained and unstained sperms were considered as dead and live respectively. About 200 Sperm were counted for both live (whitehead), partial dead (light pink head), and dead (dark pink head) in different fields. The percent viability was calculated by the formula:

$$\text{Sperm livability (\%)} = \frac{\text{No. of live sperm}}{\text{Total sperm}} \times 100$$

2.5.3 Plasma Membrane Integrity (Jeyendran *et al.*, 1984)

Sperm membrane integrity quality was evaluated by the hypo-osmotic swelling test (HOST) procedure. The solution was prepared and 50 µl of semen was mixed with 1.0 ml of hypo-osmotic solution and incubated at 37^{o} C for 1 hr. A drop of sample was examined under a bright-field microscope of 400 magnifications for curled and uncurled tail spermatozoa. About 200 curled and uncurled spermatozoa were counted for each sample. The percentage number of curled tail spermatozoa was determined and the resultant figure was taken as the HOS-reactive sperm.

2.5.4 Acrosome integrity (Watson 1975)

Giemsa stain was used to assess the acrosome integrity of the spermatozoa. A smear (10 µl) diluted and undiluted semen sample were prepared on a clean glass slide, air-dried, and fixed in 2 % glutaraldehyde solution for 30 minutes. After 30mins, the fixed slides were air-dried and the smear was incubated in Giemsa working solution for 2 hr. The slides were removed from the stain, rinsed quickly in DDW, air-dried, and examined under oil immersion (x1000) of the bright field microscope. At least 200 spermatozoa with intact acrosome and damaged acrosome (partially or completely) from each slide were counted in different fields. The percent acrosome integrity was calculated as:

$$\text{Acrosome integrity (\%)} = \frac{\text{No. of sperm with intact acrosome}}{\text{Total sperm}} \times 100$$

2.6 Fertility trial evaluation

2.6.1 Fertility Assessments of freshly diluted cross-bred Fulani chicken semen

Dilution rate 1:2 and 1:3 were selected based on the microscopic semen analysis. Fertility trials were conducted at Institute of Agricultural Research and Training, Obafemi Awolowo University, Ibadan (IAR & T) poultry breeding research farm. Semen was ejaculated and pooled from three cocks. Pooled semen was divided into five parts and diluted at 1:2 and 1:3 with both extenders. All parameters were determined on the aggregate of eggs collected per treatment throughout the three weeks egg collection periods.

2.6.2 Incubated Egg fertility Assessment

The fertilizing capability of un-extended and extended semen samples was assessed by intravaginal insemination of the hens with 0.05ml of spermatozoa for 3 weeks. Eggs were collected daily, stored for seven days, and incubated weekly. The fertility rate was determined by candling 16 days after the start of incubation. The percentage of fertility was determined using the formula below:

$$\textbf{Fertility (\%)} = \frac{\textit{no of fertile eggs}}{\textit{total no of eggs set}} \text{ x } 100$$

3.0 Results and Discussion

3.1 Results

3.1.1 Effects of Different Dilution Rate on Rooster Semen Extended with Tris Egg-Yolk Orange Juice Extender

Microscopic semen quality parameters of neat rooster semen diluted with Tris egg-yolk orange juice extender is presented in Table1.0. Percentage motility showed no significant difference ($P>0.05$) among diluted semen and neat semen inclusive. Percentage viability showed that treatment 1 had significant ($P<0.05$) lowest viability of 89.67% compared to other treatments 2 - 4 (92.67%, 96.33%, and 96.00%). However, treatment 2 to 4 (diluted semen) is not significantly different from each other ($P>0.05$). Membrane and acrosome integrity were not significantly different ($P>0.05$) among the different dilution ratios with neat semen inclusive. Membrane integrity had the same value of 93% in all the treatments while acrosome integrity had values ranged from 92.67% to 95.67%. This result implies that the membrane and acrosome integrity of rooster semen is not significantly affected by dilution rate, most especially when assessed or used immediately

3.1.2 Effects of Different Dilution Rate on Marshal Rooster Semen Extended with Tris Coconut-water Orange Extender

Microscopic semen quality analysis of marshal rooster semen diluted with Tris Coconut-water Orange juice Extender is presented in Table 1.0. Rooster semen diluted with different rates of TCWO extender was not significantly different (P>0.05) in spermatozoa motility, viability, and membrane integrity. Moreover, the same values were recorded for motility (91.67%) and Membrane Integrity (93.00%), while treatment 3 (1:3) dilution ratio had the highest percentage of live sperm (94.00%) among all treatments. Acrosome Integrity also revealed that treatments 1 and 2 had the same significant (P<0.05) higher value of 95.00% compared to treatments 3 (92.33%) and 4 (91.00%).

3.1.3 Eggs Fertility evaluation of hens inseminated with different diluents type and dilution rate

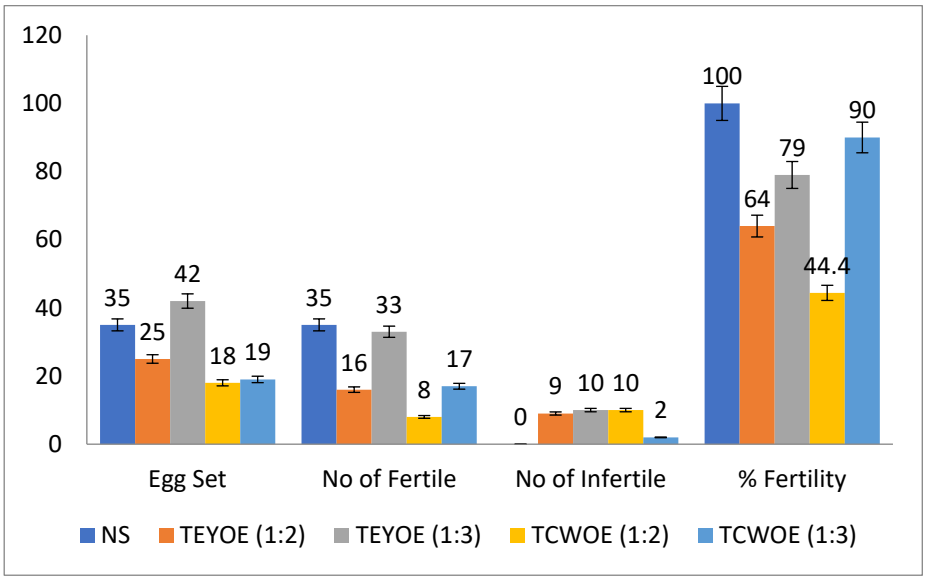
Fertility result of crossbred Fulani chicken strain inseminated with (1:2) and (1:3) diluted cock semen was presented in Fig 1. The result indicated that the 1:3 dilutions rate had higher fertility values (79.00% and 90.00%) compared to 1:2 dilutions fertility values (64.00% and 44.40%) with both extenders. Moreover, only tris coconut-water orange juice extender (1:3) dilution had a better fertility value of 90.00% compared to the value of neat semen (100.00%).

Table 1: Effects of Dilution Rate on Rooster Semen Extended with Tris Egg-Yolk orange juice Extender

Parameters	T1 (neat semen)	T2 (1:2)	T3(1:3)	T4(1:4)	SEM
Motility (%)	90.00	91.67	93.33	90.00	0.89
Viability (%live sperm)	89.67 ^{b}	92.67 ^{ab}	96.33^{a}	96.00 ^{a}	1.08
Membrane Integrity (%)	93	93	93	93	0.67
Acrosome Integrity (%)	95.67	95.33	95.67	92.67	0.70

Table 2: Effects of Dilution Rate on Extended Rooster Semen with Tris Coconut water Orange juice Extender

Parameters	T1 (neat semen)	T2(1:2)	T3(1:3)	T4(1:4)	SEM
Motility (%)	61.67^{b}	88.33^{a}	86.67^{a}	86.67^{a}	3.53
Viability (%live sperm)	53.33^{b}	86.67^{a}	86.33^{a}	91.67 ^{a}	4.65
Membrane Integrity (%)	33.50^{b}	87.33^{a}	87.66^{a}	78.33^{a}	6.91
Acrosome Integrity (%)	70.00^{b}	86.00^{a}	84.33^{a}	82.67^{a}	2.12



NS- neat semen, TEYOE- tris egg yolk orange juice extender, TCWOE- tris coconut-water orange juice extender

Fig 1: Effects of dilution rate and extender type on eggs fertility.

3.2 Discussion

3.2.1 Effects of extenders and dilution rates on microscopic semen quality

These findings however revealed that immediate dilution of rooster semen up to 4 dilution rates may not expose the sperm cells to danger for on-farm insemination use. The result obtained is in agreement with the previous studies on Punjab brown II rooster semen (Balogun *et al*., 2017; Balogun, 2019). Similarly, McDaniel *et al*. (1998) reported that dilution of semen samples 3- to 5-fold positively influences the highest SQI values. As previously reported, sperm concentration, viability, and motility collectively have a correlation with the sperm quality index (SQI), and these semen quality parameters are each very crucial in the fertilization process (Parker *et al*., 2000).

However, higher dilution of rooster semen above a 1:4 dilution ratio may not be favorable for artificial insemination use. Apparently, excessive semen dilution not considering sperm concentration require for the fertility process to take place may alter semen quality yielding a sperm quality index (SQI) that cannot be used to predict fertility (Parker and McDaniel, 2003).

Similar results were reported by (Balogun 2019) on the standardization of egg-yolk concentrations for rooster semen dilution and preservation. Also, our present observation is also corroborated by previous studies in turkey semen (Venkatesh 2005; Iaffaldano *et al*., 2010). Similarly, Keerthy *et al*., (2016) observed that the dilution rate did not have any significant effects on the storage period, the extenders, and the guinea fowl varieties.

3.2.2 Effects of extenders and dilution rates on fertilizing ability of diluted semen

The fertility trial surprisingly revealed that 1:3 dilution rates had better fertility values compared to 1:2 dilutions in both extenders. This is an indication that extending chicken semen beyond three folds dilution does not have adverse effects on the fertility rate of hen, provided the extender is void of any harmful substance to sperm cells. Siudzinska and Lukaszewicz, 2008; Boucif *et al*., 2011; Udeh and Oghenesode, 2011 reported that a good extender is similar in function to seminal plasma for all animals, simulating a natural medium for sperm. Convincingly the fertility rate is a reflection of good microscopic semen quality analysis results. This implies that microscopic semen quality analysis is a reliable test to predict the fertilizing ability of extended poultry semen, provided the extender has preservation ability. Conversely, Peterson *et al.* (2007) reported that membrane integrity is not a good predictor for fertility while Parker and McDaniel (2006) reported that a better sperm quality index which includes membrane integrity is an indication of better fertility and hatchability usually at low semen dilutions. Ultimately the dilution rates used in present these studies are not excessively high.

Convincingly, both extenders exhibited the trend of fertility records we have recorded during the standardization of both extenders irrespective of dilution rate (Balogun 2014; Balogun *et al*., 2017 a,b; Balogun *et al*., 2018; Balogun 2019). However, an improvement fertility rate was observed in both extenders, most especially with the TCWO extender. This improvement may be attributed to the antioxidant and free radicals scavenging capacity of the supplement orange juice. Moreover, a significant improvement with TCWO extender maybe its richness in energy substrates which can effectively generate ATP needed for metabolic activities of sperm cells both endogenously and exogenously to support progressive motility and viability, encourage locomotion ability the sperm in the female genital tract to the infundibulum ditto penetration of the cumulus oophorus to effect fertilization of the ova. In addition, complementarily activities of both ingredients being from plant source may also be a contributing factor to its performance compared to TEYOE.

Furthermore, our results revealed TCWOE 1:3 dilution had a closed fertility rate compared to neat semen and clearly confirmed that TCWOE is a better extender compared to its counterpart and has the potential of matching with the fertility rate of un-extended semen with further slight modification. Balogun *et al*. (2017) reported similar results on coconut water extender (CWE) and tris coconut water dried garlic extender (CWDGE). The CWE and CWDGE exhibited a good fertility value of 75% and 80% respectively compared to neat semen (NS). Also reported Ogbu *et al*., (2014) reported highest fertility of 72.10 ± 1.55 observed in eggs of hens inseminated with semen diluted heated coconut milk (HCM). Finally, our low fertility results recorded in 1:2 dilutions in this study may be attributed to irregularity in egg-laying of the crossbred Fulani domestic hens during the three weeks of fertility trials.

4.0 Conclusion

Conclusively 1:3 dilution rate is effective for poultry semen dilution and TCWOE as an extender for poultry semen dilution use has greater potentials for better and optimum fertility in hens comparably to neat semen.

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