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Effects of Diluents on Cock Spermatozoa Motility, Viability, Fertility and Hatchability

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Abstract
The study evaluated the effects of coconut water + egg yolk (CW- EY), egg yolk citrate (EYC) and skimmed milk (SM) diluents on spermatozoa motility, viability, egg fertility and hatchability in chicken. A total of 84 mature birds (48 hens and 36 cocks) randomly distributed to 4 treatments (T_1 –T_4) were used. T_1 was raw semen, while T_2 –T_4 were diluted with coconut water + egg yolk, egg yolk citrate, and skimmed milk respectively. The birds were fed a 16.5 CP and 2.60 energy layers' diet. The cocks were evaluated for the basic semen quality indices prior to the experiment. Raw semen samples were then diluted with each diluent and subjected to microscopic examination for individual progressive motility and viability every 10 minutes for 60 minutes. Semen samples were pooled from 3 cocks in each treatment and diluted in the ratio of 1:3. Aliquots - 0.1ml raw semen (T_1) and 0.25ml diluted semen ($T_2 = T_4$) were drawn and deposited into the reproductive tract of the hen. 240 eggs were incubated and candled. The result showed significant ($P < 0.05$) effects of diluents on spermatozoa individual motility. T_2 and T_3 were statistically not significant ($P > 0.05$), and were comparable with T_1. T_4 had the least (70.75) PSM value at 20 – 60min post dilution. Percentage fertility (PF) was significantly ($P < 0.05$) affected by diluent. T_1 and T_2 had higher (95.37 and 94.80) PF than T_3 and T_4. The number of infertile eggs (NI) was significantly ($P < 0.05$) affected by diluent. T_3 and T_4 had higher (14.33 and 18.33) NI than T1 and T_2. The number of dead embryos was not affected by diluent ($P > 0.05$). Percentage hatchability (PH) was significantly ($P < 0.05$) affected by diluent. T_2 had the highest PH (97.59) than T_3(94.49) and T_4 (90.24).T_1 had the least (98. 89) PH result. The result indicated significant ($P < 0.05$) effect of diluent on the percentage viable spermatozoa (PV). PV was higher in T_1 (99.00 and 98.00) than T_2 (97.75 and 96.50) and T_3 (97.50 and 95.25) and lowest in T_4 (77.75). T_2 and T_3 compared favorably with T_1 and least (77.75) in T_4 from 20 – 60minutes post dilution.

Keywords: Semen, Diluents, Motility, Viability, Fertility and Hatchability

Introduction

Native chicken has potential for improvement. Most developing countries use high yielding European commercial lines to upgrade the local fowl to increase meat and egg production traits (Mahendra, 2016). For instance, Rhode Island Red, White Leghorns, Black Australorp, Dehlem Red, Ross 308, and other synthetic breeds have been crossed with the local chickens to improve productivity (Chatterjee *et al*., 2007, and Mahendra, 2016). The use of exotic female lines and the development of synthetic breeds that are sexually dimorphic in upgrading programme may likely make artificial insemination a critical component of breeding in chicken. Assessment of diluted and undiluted cock semen revealed that application of diluent is essential for sustenance of semen quality (Hafez, 1984). Coconut water is a cheap and readily available diluent that has been used in Iran (Kewillaa *et al.,*2013) and has also been combined with egg yolk for ram semen (Dwitarizki *et al*., 2015) and recommended for cock semen (Khaezuddin and Srimaharani, 2019). Egg yolk is a good energy source and contains nearly the same biochemical and physical elements as sperm (Daramola *et al*., 2016). Skimmed milk contains lactose and milk protein which buffer the semen pH (Perca *et al*., *2017*), and has been recommended as an excellent diluent (Rahman *et al*., *2018*). In view of the international demand for natural medicinal sources for semen dilution (Ahmed *et al.,* 2020) and the current local chicken upgrading programme in Nigeria, coconut water, egg yolk citrate and skimmed milk diluents were chosen for the purpose of this study.

Materials and Methods

Study area

The research was carried out at the Poultry Unit, of the Teaching and Research Farm, Michael Okpara University of Agriculture, Umudike, Abia State. Umudike lies on co-ordinates 05° 29' N and 07° 33' E, and an altitude of about 122m above sea level. The average annual rainfall ranges from 1700 to 2100 mm. Minimum and maximum temperature are in the ranges 18 - 23°C and 26 - 36°C respectively; while relative humidity is 57- 97% (NRCRI, 2017).

Management of experimental birds

A total of 84 mature (52 weeks) birds comprising 48 Rhode Island Red laying hens and 36 local cocks were used for the experiment. The hens were sourced from a commercial poultry farm at Owerri, Imo State, while the Cocks were sourced from Ndoru market, Ikwuano Local Government Area (LGA), Abia State. The birds were housed in well ventilated netted deep litter pens and fed compounded layers' mash containing 16.5% CP and 2.6 Kcal/kg- Metabolizable energy throughout the experimental period. The birds were randomly assigned to 4 treatment groups (T_1- Raw semen, T_2- Coconut water + egg yolk, T_3- Egg yolk citrate, and T_4 – Skimmed milk) with 3 replicates per treatment with 4 hens and 3 cocks in each replicate respectively. The experiment lasted for 10 weeks. The composition of the diet is shown in Table 1.

Experimental Procedure and Data Collection

Preparation of the experimental diluents

Coconut water – egg yolk diluent

Mature coconut was carefully washed and peeled to the hard nut. The hard nut was gently cracked and the water emptied into a beaker. The coconut water was sieved to remove impurity and heated for 10 minutes in a 60ml beaker. It was allowed on the bench to cool at room temperature. Fresh egg was washed with water, dressed with 70% alcohol and dried. The egg was opened with a sterile knife and the yolk separated from the white with a yolk separator. The yolk membrane was punctured with a sterile needle. The yolk was allowed to drain into a sterile measuring cylinder. Exactly 15ml of coconut water was drawn with a syringe and added to 5ml of egg yolk in a clean beaker. Then, 135 milligrams of streptomycin was weighed and added to mixture. The entire mixture was homogenized by shaking gently.

Skimmed milk diluent

Exactly 100ml of skimmed milk was heated at 92°C for 10 minutes in a Pyrex and then poured into a clean dry beaker leaving the albumen behind. Exactly 15ml of the cooled skimmed milk was drawn with a syringe into another clean dry beaker. A 1milligram of streptomycin was added to the mixture and homogenized by shaking gently (Geoffery *et al.,* 1989).

Egg yolk citrate diluent

A 2.9g of Sodium citrate was weighed with an electronic balance in a Petri dish and added to 100ml of double distilled water in a measuring cylinder. Each five parts of the buffer mixture was mixed with 1 part of egg yolk prepared as previously described. The final mixture was homogenized by shaking gently. The pH was adjusted to 7.4.

Semen collection, dilution and evaluation

The cocks were trained for semen collection twice weekly for two weeks using abdominal massage technique as described by Burrows and Quin (1937). Raw semen samples were collected from 6 cocks in each treatment, diluted with 0.9 saline and evaluated for volume, motility, sperm concentration, live/dead spermatozoa, normal/abnormal sperm and total sperm in ejaculate prior to the experiment to determine insemination dose for each treatment. Cocks that had semen volume up to 0.4ml, 85% motility score, and $4.6x10^6$ sperm concentration were selected for the experiment. Thereafter, semen samples were pooled from three cocks in each treatment into four clean beakers accordingly. A 0.2ml of raw semen was drawn from each pooled semen sample and diluted with 0.2ml of each diluent and mixed gently. A drop of the diluted semen was placed on a clean pre-warmed microscope slide, clipped under a light microscope and viewed at (x400) magnification every 10min for 60min. Progressive motility was determined by subjective scoring in percentage. Percentage live spermatozoa proportion was determined by differential staining technique. A drop of diluted semen sample was placed on a clean dry slide with a stirring rod and two drops of eosin-negosin added to it with a dropper. A smear was made by placing a warm slide over the first, spreading the mixture evenly by pulling the two slides apart and placing quickly on a warming device to prevent cold shock as the two slides dry. The slides were clipped to a light microscope and observed at x400 magnification for the number of live and dead sperm. Spermatozoa which picked up the stain were considered dead, while those that exuded the stain were considered alive. Exactly 100 sperm cells were counted in each slide and classified as alive or dead at the time of staining. The live and dead sperm were reported in percentage (Bearden *et al.,* 2004).

Artificial Insemination

The hens were sexually stimulated by "venting" as described by Hafez (1984). A 1ml of raw semen was diluted with 2ml of each diluent and maintained warm in warm water bath. All the syringes and the diluents were maintained warm in the water bath. An insemination doses of 0.1ml/bird for of fresh (T_1) and 0.25ml/bird for diluted semen (T_2 –T_4) containing 2 million spermatozoa were pooled and deposited into the vagina (5cm depth) of each female within 30 minutes of collection using a clean, dry plastic syringe. The hens were inseminated 2 times weekly during the late afternoon throughout the experimental period of 10 weeks.

Egg collection, incubation and candling

Eggs were collected daily from hens in each treatment, properly identified, and stored in an airy environment in egg crates. The eggs were set for incubation every four

days and incubated with a locally made incubator. The set eggs were candled on the 10th day of incubation. Egg breakout was done after (21 days). Eggs that did not hatch were recorded as dead embryos.

Experimental design and statistical analysis

The experiment was arranged a CRD. Data collected were subjected to analysis of variance (ANOVA) (Steel and Torrie, 1980). Means were separated using Duncan' multiple range test (Duncan, 1955). The linear statistical is as shown thus:

$Y_{ij} = +\sigma i + e_{ij}$

Where:

Y_{ij} = Single observation, = overall mean, σ = Effect of diluents, e_{ij} = Random error

Results and Discussion

Effect of diluents on sperm progressive motility is shown in Table 2. The result obtained from the present study showed significant ($P < 0.05$) effect of diluent on percentage spermatozoa motility (PSM). PSM was higher ($P < 0.05$) in T_1 (98.25) than T_2 (95.75) and T_3 (94.25) and least in T_4 (97.25) within the first 0-10minutes post dilution. The mean PSM values did not follow a definite pattern, T_2 and T_3 diluents competed ($P > 0.05$) favorably with T_1 from 20-60 minutes post diluent. T_4 had the least PSM mean values from 0-60 minutes post dilution. The result obtained in the present study showed a general progressive decline in PSM as holding time increased from 0-60 minutes. The result obtained in this study was in agreement with those of Ogbu *et al.* (2014), Siti and Miyayu (2019) and Daramola *et al*. (2016) who reported better motility of cock and ram spermatozoa in coconut water and coconut water + egg yolk diluents respectively. The reason for the result obtained for T_1 from the initial 0-10 minutes post dilution was in agreement with Packer and McDaniel (2006) who reported that diluting semen with physiological saline causes hyper excitation, resulting in initial increased vigorous motility which leads to exhaustion and subsequent reduced motility. Similarly, variations in the viscosity of the diluents and cold storage were implicated for the differences in the PSM obtained in this study. Physiological saline, being less viscous than the others had higher initial PSM. In the other studies, the semen samples were frozen as opposed to the present study. Donoghue and Wshart (2000) reported that cold storage reduces sperm motility in poultry due to ultra structural damage to spermatozoa tail during freezing and thawing. The mean PSM values obtained in this study were much higher than those (71.60 ± 1.59, 65.55 ± 1.63, 30.85 ± 2.39, 68.60 ± 1.02 and 43.40 ± 5.10) reported by Ogbu *et al.* (2014) for Na-citrate, heated coconut milk, unheated coconut milk and normal saline respectively. Similarly, PSM mean values recorded in this study across the treatments were higher than 77% reported by Lubis (2011), Damang *et al*. (2012) and Wiyanti *et al.* (2013), and 68.3% reported by Tethool *et al.* (2017), respectively. However, the result of this study was comparable with 89% and 83.7% reported by Indrawati *et al.* (2013). The reason for the result obtained in this study for T_2 and T_3 could be attributed to the inherent antioxidant properties and the energy rich substrates of the diluents (Santos *et al.* 2013). Similarly, Siti and Miyayu (2019) reported that coconut water + egg yolk conjugate diluent significantly ($P < 0.05$) increased cock sperm progressive motility more than coconut + fructose diluent. This is because, coconut water and egg yolk contain glutamic acid and the major electrolytes which are found in the seminal plasma of cock semen that are used as energy sources by sperm cells in vitro (Aboagla and Terada, 2004; Siudziuska ad Lukaszewics, 2008). The effect of diluents on percentage live/viable spermatozoa is shown in Table 3.

The result of this study indicated significant ($P < 0.05$) effect of diluent on the percentage viable spermatozoa (PV). PV was higher in T_1 (99.00 and 98.00) than T_2 (97.75 and 96.50) and T3 (97.50 and 95.25) and lowest in T1 (95.25 and 93.00) within the initial 0-10 minutes post dilution. T_2 and T_3 compared favorably ($P > 0.05$) with T_1 without any definite pattern from 20-60 minutes post dilution, T_4 differing significantly ($P < 0.05$) with the least (77.75) PV mean values from 0-60 minutes post dilution. PV mean values decreased progressively with increase in holding time. The result obtained for PV in this study agreed with previous reports; Ogbu *et al. (*2014), and Khaeruddin and Srimaharami, (2019) for coconut milk and coconut water respectively. PV mean values obtained in this study for T_2 and T_3 diluents were similar to 99.73 ± 0.13 and 96.33 reported by Balogun *et al.* (2019) and Khaeruddin and Srimaharani (2019), respectively, but higher than 79.94 and 83.87 reported by Lubis (2011) and Tethool *et al.* (2017) for cock semen in coconut water based diluents. The reason for the result obtained in this study could be attributed to the fact that coconut water and egg yolk are rich in energy and electrolytes which effectively supplied the ATP required for endogenous and exogenous metabolic activities and prolonged motility and viability (Balogun *et al*., 2020) and as well, maintained the electrolyte balance in semen (Reddy and Lakhsmi, 2014). Although Skimmed milk has been reported to contain lactose and milk protein which buffers the semen pH (Perca *et al*., 2017), and has been recommended as a more excellent semen diluent than Tris based extender (Rahman *et al*., 2018), and because antibiotic was added against bacterial contamination, high viscosity was implicated for its low performance in the parameters measured in this study. The result of the present study corroborated those of Daramola *et al.* (2016), Sit and Miyayu (2019) and Sun *et al*. (2019) who reported significant ($P< 0.05$) higher percentage live spermatozoa of buck and cock in coconut water based diluent respectively. The effect of the extenders on the fertility of the Cock semen was also evaluated and represented in Table 4.

The result obtained in this study did not show any significant ($P > 0.050$) effect of diluent on the number of fertile egg among the treatments. However, NI was

significantly ($P < 0.05$) higher in T_4 (18.33) and T_3 (14.33) than T_1 (3.67) and T_2 (14.00). PF was significantly ($P < 0.05$) higher in T_1 (95.37) and T_2 (94.80) than T_3 (81.65) and T_4 (76.50). The number of dead embryos and hatched eggs did not differ ($P > 0.05$) among the treatments. PH was significantly ($P < 0.05$) affected by diluent. T_2 had higher (97.59) PH than T_3 (94.49) and T_1 (90.24), but significantly ($P < 0.05$) lowest in T_4 (80.89). The result of the present study was in agreement with those of Balogun *et al.* (2020) who reported 95% fertility in hens inseminated with coconut water based cock semen diluents. The results obtained in T_2, T_3 and T_4 were higher than 72.10 ± 1.55 and 76.50 ± 1.20, 66.90 ± 2.33 and 70.50 ± 1.35, 34.90 ± 3.14 and 51.40 ± 1.16, 52.50 ± 1.58 and 65.60 ± 2.40 and 51.80 ± 1.87 and 54.00 ± 2.09 reported by Ogbu *et al.*(2014) for Na-citrate, heated coconut milk, unheated coconut milk and saline respectively. The differences between the two studies could be due to the effect of cold storage on the spermatozoa. Parkinson (2009) reported that frozen storage may advance the maturation of sperm membrane, increase the proportion of capacitated and acrosome – reacted cells; resulting in reduced viability, fertility and distorted embryonic development after AI. Similarly, Daramola *et al.* (2016) cryopreserved ram semen in coconut water diluents for 30 days and reported higher proportion of induced acrosome reacted sperm cells compared to the control. Siti and Miyayu (2019) reported drastic deceases in sperm motility and viability as low as 40% and 39.5% after 7days of cold storage in coconut water diluents respectively, which could lead to reduced fertility. The decline in the semen quality after cold storage could be attributed to the unique physiological characteristics of avian spermatozoa which limit the preservation of avian semen (Donoghue and Wishart, 2000; Thurston and Hess, 1987). It therefore appears that coconut water diluent is better used immediately after dilution. Also, variations in the composition of the diluents could be implicated for the differences in the results. In the present study, egg yolk was added to coconut water which could have led to higher fertility values recorded in this study. It was also observed that fertility and hatchability results were higher and comparable in the diluents that had better motility and viability mean values. This is because sperm viability determines competitive fertilization success (Kumaresan *et al*., 2017). The reason for the results obtained in this study for T_2 and T_3 could be due to the ability of the diluents to supply the requisite electrolyte balance, protect the fibroblast of the sperm cells as well as the ATP which potentiated viability, capacitation, acrosome reaction and locomotive capacity of the spermatozoa to swim-up the infundibulum (Blanco *et al*., 2011; Reddy and Lakhmi, 2014; Kumaresan *et al*., 2017 and Baice *et al*., 2018). Although high levels of fertility and hatchability results were obtained in this study, T_2 and T_3 diluents appeared to be better than T_4.

Conclusion

The result of this study showed significant effect of diluents on individual spermatozoa motility and viability, percentage fertility and hatchability of fertile eggs. Coconut water and egg yolk- citrate diluents performed better than skim milk and compared favourably with raw semen in almost all the parameters measured. Based on the study therefore, coconut water + egg egg yolk citrate and egg yolk citrate were recommended for on-farm artificial insemination.

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Table 1: Percentage composition of the diet

Ingredient	Composition (%)
Maize	25.0
Soya bean meal	5.0
Wheat offal	48.10
Groundnut cake	10.0
Bone meal	3.0
Limestone	7.0
Lysine	0.75
Methionine	0.60
Salt	0.30
Premix	0.25
	100.00

Calculated value: CP (%) = 16.5, Energy (Kcal kg-1) = 2.60, CF (%) = 5.7

Table 2: Effect of diluents on sperm progressive motility

Parameters	Time	Raw semen	CW- EY	EYC	SM	SEM
Progressive motility (%)	0 min	98.25^{a}	95.75^{b}	94.25^{c}	90.00^{d}	0.79
(0-100)	10 min	97.75^{a}	94.75^{b}	92.50^{b}	87.50^{c}	1.05
	20 min	95.75^{a}	91.75^{ab}	90.00^{b}	84.75^{c}	1.23
	30 min	93.75^{a}	90.50^{ab}	88.50^{b}	83.25^{c}	1.14
	40 min	90.25^{a}	87.50^{ab}	85.50^{b}	79.25^{c}	1.23
	50 min	87.50^{a}	85.00^{a}	82.00^{a}	75.75^{b}	1.41
	60 min	81.50^{a}	81.50^{a}	78.25^{a}	70.75^{b}	1.42

^{a,b,c} Means with different superscripts across the rows differ significantly at P < 0.05; CW -EY = Coconut Water + Egg Yolk Diluent, EYC = Egg Yolk Citrate Diluent, SM = Skimmed Milk Diluent, SEM = Standard Error of the Mean

Table 3: Effect of diluents on live / viable spermatozoa

Parameters	Time	Raw semen	CW - EY	EYC	SM	SEM
Live/Viable Sperm %	0 min	99.00^{a}	97.75^{ab}	97.50^{ab}	95.25^{b}	0.54
	10 min	98.00^{a}	96.50^{ab}	95.25^{ab}	93.00^{b}	0.69
	20 min	96.25^{a}	95.25^{a}	93.00^{a}	89.25^{b}	0.88
	30 min	95.50^{a}	93.50^{a}	91.50^{ab}	87.25^{b}	1.01
	40 min	93.50^{a}	91.50^{ab}	88.75^{b}	84.25^{c}	1.11
	50 min	90.50^{a}	89.25^{ab}	85.25^{bc}	82.00^{c}	1.11
	60 min	85.75^{a}	87.00^{a}	84.75^{a}	77.75^{b}	1.22

^{a,b,c} Means with different superscripts across the rows differ significantly at P < 0.05; CW - EY = Coconut Water + Egg Yolk Diluent, EYC = Egg Yolk Citrate Diluent, SM = Skimmed Milk Diluent, SEM = Standard Error of the Mean

Table 4: Effect of diluents on fertility and hatchability of Rhode Island Red hens' egg

Parameters	Raw	CM	EYC	SM
No of set	80.08	80.33	80.31	80.32
SEM	5.78	5.78	5.86	5.89
No of fertile	76.33	76.00	65.66	61.67
SEM	5.78	6.66	7.44	8.01
No of infertile	3.67^{b}	4.00^{b}	14.33^{a}	18.33^{a}
SEM	0.33	1.00	2.33	2.73
% fertile (%)	95.37^{a}	94.80^{a}	81.65^{b}	76.50^{b}
SEM	0.57	1.51	3.63	4.54
No DE	7.67	2.00	3.33	11.67
SEM	3.28	1.15	2.40	3.48
No hatched	68.67	74.00	62.33	50.00
SEM	4.37	5.51	8.84	8.08
% hatchability	90.24^{ab}	97.59^{a}	94.49^{ab}	80.89^{b}
SEM	3.59	1.32	3.84	5.88

^{a,b,c} means with different superscripts across the rows differ significantly at P<0.05; CMD = Coconut Milk Diluents; EYCD = Egg Yolk Citrate Diluents; SMD = Skim Milk Diluents; SEM = Standard error of the mean