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Evaluation of Egg Yolk Plasma as Replacement for Whole Egg Yolk in Chicken Semen Extender

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ABSTRACT

Poultry semen has high spermatozoa concentration and needs to be extended (diluted) for efficient artificial insemination; extenders containing egg yolk often have various limitations to their use. This study assessed chicken egg yolk plasma (EYP) as replacement for chicken whole egg yolk (EY) in semen extenders. The preservative potential under room and cold storage and its influence on fertility and hatchability in breeder chicken flock was also assessed. Ten broiler breeder cocks and one hundred hens were used for the study. Semen ejaculates from the cocks were pooled and divided into five portions. One portion each was extended with egg yolk plasma (EYP); phosphate buffer saline (PBS); egg yolk (EY) +PBS or EYP +PBS while the fifth unextended portion termed fresh undiluted semen (FUS) served as the control. The ratio of EYP and EY to PBS was 1:4. The semen samples were evaluated at 1, 2 and 3 hours after extension under room temperature and at 24, 48 and 72 hours after cold storage (4°C). The hens were inseminated with the freshly extended semen or with cold preserved semen, each for three weeks. Fertility and hatchability of the eggs were recorded on 18th and 21st day, respectively post setting. At room and cold storage temperatures, the quality parameters of the semen significantly reduced as the holding time increased. The EYP groups had the highest spermatozoa motility of 55.33% at 72 hours of cold storage. Freshly extended semen with EYP+ PBS had higher quality and fertilizing potentials which resulted in increased egg fertility (87.49%) and hatchability (84.95%). Cold preserved EYP + PBS semen resulted in significantly higher fertility (75.66%) and hatchability (90.90%). It was concluded that egg yolk plasma could conveniently replace whole egg yolk in chicken semen extender resulting in improved sperm viability and egg hatchability.

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Introduction

Poultry is currently the most commercialized livestock in the world. Profitable poultry farming depends on quality chicks, feeds and good management. To produce enough chickens for the increasing demand for meat and egg, healthy broiler and layer chicks must be produced. Many commercial poultry breeder farms employ artificial insemination (AI) technique for the production of chicks. This technique has the advantage of utilizing one cock to inseminate up to 30 hens while in natural mating one cock only serves 8 to 10 hens per day (Thatohatsi, 2009). The cocks' ejaculate is generally low in volume but highly concentrated; extending it with suitable diluent at specific rates prior to AI or storage will improve poultry production. Sperm motility and fertilizing ability generally deteriorates within 1 hour after collection when not properly stored *in-vitro* (Dumpala et al., 2006).

The chicken egg yolk is generally accepted as an effective agent in semen extenders for protection of spermatozoa against storage cold shock and the lipid-phase transition effect (Aboagla and Terada, 2004). This primary benefit derived from fresh egg yolk is attributed to its low density lipo-protein and lecithin contents (Noussa et al., 2002; Watson, 1981). Egg yolk contains two major fractions when centrifuged: solid and plasma; and the low density lipo-protein and lecithin which confer the protective ability on egg yolk has been traced primarily to the plasma portion (Pace and Graham, 1974; Bergeron et al., 2004). In previous studies that evaluated use of egg yolk as an extender component, bulkiness and high affinity for spoilage microorganisms reduced its usefulness, especially for short term semen storage (Sexton, 1977; Aurich et al., 1997). The egg yolk plasma is devoid of this

major shortcoming. Thus, this study seeks to evaluate the use of egg yolk plasma as replacement for egg yolk in cock semen extender; and its effect on storability and fertilizing ability of the semen and hatchability of fertilized chicken eggs.

Materials and Methods

Experimental Location, Animals and Management

Thirty- two (32) weeks old broiler breeder cocks (10) and hens (100) of Marshall R. breed managed at the broiler breeder unit of Osin Farms Nigeria Limited, Yakooyo, Osun State, Nigeria were used for this study. The laboratory analyses were carried out in Animal Reproduction Laboratory, Department of Animal Sciences of Obafemi Awolowo University, Ile-Ife, Nigeria. The cocks were housed individually while hens were caged in groups of two in battery cages inside a wire net sided building. The birds were fed diets containing 18% crude protein and 2.750 Kcal/Kg ME. Clean water was provided ad-libitum.

Preparation of Semen Extenders

Egg yolk plasma (EYP) was obtained by carefully separating chicken egg yolk from the albumen. The egg yolk was collected into a glass beaker and equal volume of distilled water was added. The mixture was vortex mixed for 10 minutes and then centrifuged at 3.500 rpm for 20 minutes. The liquid plasma was carefully decanted and refrigerated for use within 24 hours. Phosphate buffer saline (PBS) was prepared according to the method of Medicago (Medicago, 2010), Sodium chloride (0.8 g), potassium hydrogen phosphate (0.114 g), glucose (0.03 g) and streptomycin (0.05 g) were dissolved in 100 ml of distilled water to obtain PBS used for this study. The EY, EYP and PBS were used to prepare four different extenders for this study (Table 1).

Table 1. Experimental semen extenders

Groups	Semen Extenders
1	EYP
2	EY+PBS
3	EYP+PBS
4	PBS
5	FUS (Control)

EYP: Egg yolk plasma only; EY+PBS: Whole egg yolk with Phosphate Buffer Saline at 1:4 (v/v); EYP+PBS: Egg yolk plasma with phosphate buffered saline at 1:4 (v/v); PBS: Phosphate Buffer Saline only; FUS: Fresh undiluted semen (Control)

Semen Collection, Extension and Analysis

The first phase of this study assessed the extended semen for quality parameters before and after cold storage. Semen was collected from the ten cocks by abdominal-vent massage technique twice within one week for two weeks (first week for assessment under room temperature and second week for assessment after storage at 4℃). Each day collections were pooled and divided into five portions for the purpose of extension. Semen was extended by adding equal volumes of the semen and the respective extenders (Table 1). The semen was assessed immediately after extension and thereafter hourly for three (3) hours. This was done at room temperature. Similarly, extended semen was assessed at 24, 48 and 76 hours after storage at a temperature of 4℃. The parameters assessed were sperm motility, normal sperm cells, live spermatozoa and acrosomal integrity. Motility was assessed subjectively under light microscope (Olympus C01) and expressed as the percentage of motile sperm with moderate to rapid progressive movement. Sperm morphology and live to dead spermatozoa ratio was determined using eosin-nigrosin staining technique. Congo red with Giemsa stain was used to determine sperm cells with intact acrosome (Blesbois, 2007).

The second part of the study involved insemination of the extended semen groups. Semen was collected from the ten cocks twice weekly over a six-week period. Freshly collected semen was maintained at 37℃ for quick estimation of semen concentration and motility using the portable Handycope® microscope. Semen volumes per ejaculate ranged from 0.4-1.0 ml. Thereafter pooled semen was divided into five portions and four of these were extended with equal volumes of the four extenders (Table 1) to give semen with an average concentration of between 2.0-2.5 billion sperm cells per ml of inseminate. The extended semen groups were either used immediately to inseminate hens or stored at 4℃ for later insemination.

Estimation of fertility and hatchability of eggs obtained from hens inseminated with fresh or cold preserved extended semen

This phase of the study assessed fertility and hatchability rates of eggs obtained from hens inseminated with extended semen. Doses 0.5 ml of extended semen (4 groups) and 0.20 ml of fresh undiluted semen (control) were used to inseminate twenty hens each in the five groups within one hour of extension, twice weekly for three weeks. Eggs were collected from the inseminated birds for a total period of three weeks and incubated in a Petersime made split incubator. Total number of fertile eggs (after candling on day 18) and hatched eggs were recorded from which the percentage fertility and hatchability were estimated. A week after the last insemination, the procedures above was repeated with extended semen samples stored at 4℃ for 72 hours using the same set of hens. A total of 3.075 eggs were incubated over the six weeks insemination periods. Number of eggs set per treatment group are shown in Table 4. Eggs were aggregated from inseminated birds and stored at 18℃ over 6 days and set on the seventh day

Data Analysis

Data from the semen analyses were in four replicates (two days analyzed in two replicates) while the fertility and hatchability data were in three replicates (eggs set once a week for three weeks). For each parameter all the replicates for each treatment were combined and subjected to statistical analysis using Two-way Analysis of Variance (SAS, 2002) and means were separated with Duncan Multiple Tange Test of the same software.

Results

Fertilizing ability of extended broiler breeders' semen under room temperature and after cold storage

Fertilizing ability of broiler breeder's cocks' semen in different extenders over a period of three hours under the room temperature (around 28℃) is presented in Table 2. It was observed that both extender type and extension time had significant ($p<0.05$) effect on spermatozoa motility, spermatozoa morphology, acrosome integrity and live spermatozoa. Spermatozoa motility significantly reduced with increasing extension time for all the extenders at room temperature. Semen diluted with egg yolk plasma+PBS had the highest spermatozoa motility across the hours (60 to 78%) compared to the fresh undiluted semen, FUS (22 to 71%), which had the lowest spermatozoa motility compared to those diluted with extenders. Percent motile cells were significantly reduced in the FUS (71 to 22%) within three hours of collection compared to 78-55% in the extended semen. Across the time for each extender, the percent motility also reduced significantly ($p<0.05$) as extension time increased.

There were no differences in the percent normal spermatozoa within the extended semen groups at both 1 hr. and 2 hr. period while FUS had a lower ($p<0.05$) percent normal spermatozoon at 2 hours compared to the four extended semen groups. There was reduction in percent normal cells as the time increased and EYP+PBS group gave the least abnormal cells with higher percent normal cells of 69.09% at 3 hours. EYP+PBS group had the highest ($p<0.05$) percent intact acrosome at all the time periods (92.67-78.77%) while FUS had the lowest (83.46-65.96%). However, across the time periods for each extender, the percent intact acrosome significantly ($p<0.05$) reduced as time increased. The EYP+PBS also had the best potential of keeping more sperm cells alive than other groups. However, as time increased, there was significant reduction in the percent live spermatozoa in all the extenders. The extenders had different ability to keep the semen viable under cold storage (4℃) as shown in Table 3. The extender and storage time had significant effect on the semen quality. Quality assessment of the cold preserved extended semen samples showed a better performance than the undiluted samples. At 24 hr. spermatozoa motility was already reduced to below 50% reaching 0% at 72 hr. in the undiluted sample whereas the extended samples still maintained around 50% at 72 hr. Across the storage time for each extender, the percent motility significantly ($p<0.05$) reduced as storage time increased. This situation was also recorded for the other quality parameters measured. The EYP+PBS group gave the highest normal cells of 62.94% at 72 hr. There were no differences ($p>0.05$) among EYP, EY+PBS and EYP+PBS groups at 24, 48 and 72 hours of storage time in the percent intact acrosome. Similarly, these groups of extenders retained more than 50% lives cells even at 72 hr. (55, 50 and 55%, respectively).

Fertility and hatchability of eggs from hens inseminated with fresh or cold preserved extended semen

The result of the fertility and hatchability trials with the four types of extended semen are shown in Table 4. The hens were inseminated within 1 hr. of semen collection and extension. Percent fertility varied from 73.23±20.56% in EYP to 87.65±5.07% in PBS, while percent hatchability varied from 77.87±6.79% in PBS to 86.50±17.55% in EYP. There were no significant differences in both parameters among the semen extender groups. However, there was significant ($p<0.05$) difference in the fertility and hatchability values when the semen was extended and stored for 72 hours. EYP+PBS showed the highest percent fertility (75.86%) and percent hatchability of 90.90% similar to the 94.74% of the fresh undiluted semen. EYP had the least percent values for both fertility and hatchability (50.00% and 40.00%, respectively).

Table 2. Fertilizing ability of extended broiler breeder cock's semen at room temperature

Parameter	TRT (hours)	Extenders				
		Egg Yolk Plasma	Egg Yolk+PBS	Egg Yolk Plasma+PBS	PBS	Undiluted Semen
Spermatozoa motility (%)	1	77.00 ± 2.00^{Aa}	76.67 ± 2.67^{Aa}	78.00 ± 1.31^{Aa}	73.33 ± 2.03^{Aa}	71.00 ± 3.98^{Ab}
	2	69.30 ± 3.67^{Bab}	69.67 ± 2.91^{Bab}	71.00 ± 3.00^{Ba}	64.67 ± 2.67^{Bb}	36.67 ± 0.73^{Cc}
	3	58.00 ± 4.00^{Ca}	60.33 ± 1.33^{Ca}	60.67 ± 1.64^{Ca}	55.00 ± 3.01^{Ca}	22.00 ± 1.50^{Cb}
Normal spermatozoa (%)	1	84.81 ± 3.57^{Aa}	83.93 ± 2.69^{Aa}	85.74 ± 2.85^{Aa}	81.83 ± 2.76^{Aa}	74.44 ± 2.03^{Ab}
	2	76.36 ± 3.36^{ABa}	76.24 ± 5.88^{Aa}	78.99 ± 5.02^{Aa}	72.25 ± 10.61^{ABa}	59.44 ± 0.96^{Bb}
	3	67.13 ± 7.13^{Ba}	63.64 ± 6.99^{Bb}	69.09 ± 7.89^{Ba}	60.68 ± 9.27^{Bb}	54.20 ± 7.29^{Bc}
Cell with intact acrosome (%)	1	89.41 ± 1.66^{Aab}	87.57 ± 5.01^{Aab}	92.67 ± 0.67^{Aa}	85.28 ± 3.84^{Aab}	83.46 ± 5.89^{Ab}
	2	83.14 ± 0.33^{Aa}	80.79 ± 2.80^{Aa}	86.46 ± 0.18^{Ba}	77.63 ± 2.28^{Bab}	71.65 ± 1.45^{Ab}
	3	75.97 ± 0.96^{Ab}	75.20 ± 1.63^{Ab}	78.77 ± 1.73^{Aa}	72.08 ± 0.57^{Cc}	65.96 ± 2.07^{Bd}
Live spermatozoa (%)	1	76.67 ± 2.89^{Aab}	76.67 ± 3.57^{Aab}	80.00 ± 2.69^{Aa}	73.33 ± 2.75^{Ab}	73.33 ± 1.48^{Ab}
	2	70.00 ± 5.00^{Bab}	70.00 ± 5.87^{Bab}	73.33 ± 2.89^{Ba}	65.00 ± 5.70^{Bb}	56.67 ± 0.89^{Bc}
	3	58.33 ± 2.09^{Cb}	60.00 ± 6.99^{Cb}	63.33 ± 3.98^{Ca}	50.00 ± 9.27^{Cc}	40.00 ± 7.29^{Cd}

PBS-Phosphate Buffered Saline; $^{a-d}$ Means (±SD) within each row with different superscripts differ significantly ($p<0.05$); $^{A-C}$ Means (±SD) within each column of every parameter with different superscripts differ significantly ($p<0.05$); TRT: Time at room temperature

Table 3. Fertilizing ability of extended broiler breeder cock's semen under cold storage

Parameter	TCS (hours)	Extenders				
		Egg Yolk Plasma	Egg Yolk+PBS	Egg Yolk Plasma+PBS	Phosphate Buffered Saline	Fresh Undiluted Semen
Spermatozoa motility (%)	24	69.33±1.70^{Aa}	69.33±1.56^{Aa}	69.33±0.35^{Aa}	67.00±0.15^{Aa}	43.00±2.65^{Bb}
	48	62.00±3.91^{Ba}	55.00±3.70^{Bb}	64.00±3.14^{Ba}	50.67±0.93^{Bc}	20.67±0.56^{Bd}
	72	55.33±2.52^{Ca}	49.00±2.00^{Cb}	55.00±1.42^{Ca}	45.00±3.42^{Cc}	0.00±0.00^{Cd}
Normal spermatozoa (%)	24	74.90±3.82^{Aab}	76.96±6.40^{Aab}	80.87±5.79^{Aa}	66.13±10.61^{Abc}	59.44±0.96^{Ad}
	48	68.19±7.87^{Aa}	63.52±6.89^{Ba}	64.73±8.19^{Ba}	62.11±5.67^{Aa}	44.14±8.41^{Bb}
	72	55.60±1.81^{Bb}	56.67±2.87^{Bab}	62.94±4.23^{Ba}	57.95±3.55^{Bc}	36.81±3.79^{Bd}
Cell with intact acrosome (%)	24	86.82±1.23^{Aa}	86.11±0.96^{Aa}	88.89±0.67^{Aa}	84.64±4.02^{Aa}	68.16±4.19^{Ab}
	48	82.32±0.87^{Aa}	81.25±0.80^{Aa}	86.36±1.18^{Aa}	78.17±0.68^{Ab}	61.89±5.66^{Ac}
	72	76.31±0.53^{Aa}	75.00±1.63^{Aa}	76.90±0.74^{Aa}	73.02±0.53^{Aab}	42.55±3.78^{Bc}
Live spermatozoa (%)	24	70.00±3.82^{Aa}	70.00±6.40^{Aa}	70.00±5.79^{Aa}	65.00±10.61^{Ab}	43.33±0.96^{Bc}
	48	63.33±7.87^{Ba}	56.67±6.89^{Bb}	65.00±8.19^{Ba}	50.00±5.67^{Bb}	20.00±8.41^{Cc}
	72	55.00±1.81^{Ca}	50.00±2.09^{Cb}	55.00±4.23^{Ca}	45.00±3.55^{Cc}	0.00±0.00^{Cd}

PBS-Phosphate Buffered Saline; $^{a-d}$Means (±SD) within each row with different superscripts differ significantly ($p<0.05$); $^{A-C}$ Means (±SD) within each column with different superscripts in each parameter differ significantly ($p<0.05$); TCS: Time in cold storage

Table 4. Fertility and hatchability of eggs from hens inseminated with extended semen

Extender	Fresh semen			Cold preserved semen		
	No of eggs	Fertility (%)	Hatchability (%)	No of eggs	Fertility (%)	Hatchability (%)
PBS	315	87.65±5.07	77.87±6.79	305	53.85±0.77^{c}	78.57±0.98^{b}
EYP	300	73.23±20.56	88.50±17.55	310	50.00±0.11^{c}	40.00±1.22^{c}
EY+PBS	310	81.66±4.44	84.80±9.58	315	62.05±0.83^{b}	80.00±0.35^{b}
EYP+PBS	305	87.49±10.85	84.95±15.96	300	75.66±0.27^{a}	90.90±0.96^{a}
FUS	300	77.21±23.09	82.54±22.50	310	*63.33±0.98^{b}	94.73±1.65^{a}

$^{a-c}$ Means ±SD in the same column with different superscript are different ($p<0.05$); * Fresh undiluted semen was not cold preserved; EYP: Egg yolk plasma only; EY+PBS: Whole egg yolk with Phosphate Buffer Saline at 1:4 (v/v); EYP+PBS: Egg yolk plasma with phosphate buffered saline at 1:4 (v/v); PBS: Phosphate Buffer Saline only; FUS: Fresh undiluted semen (Control)

Discussion

Sperm motility is highly indicative of the viability of sperm and the quality of the semen sample. The motility values obtained in this study are similar to the values obtained in the fresh semen samples of New Hampshire (Chalah et al., 1999) and White Leghorn (Bah et al., 2001). It had been found that sperm motility and fertilizing ability of cock sperm generally deteriorate within 1 hour after collection (Dumpala et al., 2006). However, findings have shown that diluents or extenders can preserve sperm motility and viability (Thatohatsi, 2009). Sperm motility was found to be lower in the EY+PBS group compared to EYP+ PBS group and this shows the superior effects of the later on viability of cock semen. The liveability results from this study is lower than 91-94% live spermatozoa without any deformities reported by Van Voorst and Leenstra (1995) and Tselutin *et al.* (1996). However, 70 to 80% live normal spermatozoa after extension recorded by Siudzinska and Lukaszewicz (2008) is similar to the values obtained in this study. The semen with EYP+PBS had a significantly higher percentage intact acrosome at 3 hours under the room temperature. Percent acrosome was 45% higher in semen extended with EYP+PBS than the fresh diluted semen. According to Thatohatsi (2009), the acrosome and the mid-piece are the most sensitive regions in the cock's sperm, with the mid-piece being quicker to deteriorate than the other regions; and the connecting area between the sperm head and midpiece of poultry spermatozoon is more sensitive to external factors. In this study, fertilizing ability of semen extended with EYP+PBS compare favorably with those extended with EY+PBS.

Storage is known to be a factor that affects the quality of semen and if it is not optimum, the viability of the semen reduces instantly. The most common procedure for short-term fowl semen storage (hours to days at refrigerator temperature) requires mixing semen with suitable extender to retain their viability *in-vitro* (Siudzinska and Lukaszewicz, 2008). In this study, decrease in motility, live cells, morphologically normal spermatozoa and an increase in dead spermatozoa, spermatozoa with damaged acrosomes and coiled tails were observed with increase in storage period. This corroborates the findings of Hudson *et al*. (2016) who reported significant increase in percentage dead and abnormal spermatozoa with increasing storage time. However, semen extended with EYP+PBS had higher values than the EY+PBS group in this regard. The morphological sperm defects generally affect the fertility more than the sperm motility does. Temperature changes usually lower sperm viability through it increases sperm abnormalities (Lukaszewicz and Kruszynski, 2003). The 20-26% dead cells obtained in this work agrees with the range (14-27%) reported by Siudzinska and Lukaszewicz (2008). Species-specific differences in the ability of cock

sperm to withstand the various stresses caused by cold storage have also been identified as a major problem in the preservation of cock genetic material and biodiversity (Siudzinska and Lukaszewicz, 2008; Blesbois et al., 2001).

The test for fertility and hatchability is important because it confirms the quality of the semen used whether extended or undiluted and it provides information on the suitability of the cocks for breeding purpose. The fertility rates obtained following fresh semen insemination in the current work are in agreement with Petek and Dikmen (2006), who recorded values ranging between 61.8 and 85.4% in broiler breeders. In the domestic fowl, it has been found that the sperm numbers, type of hens (broiler or layer types) and age may affect the *in vivo* storage of the sperm and subsequently, the fertility of the eggs (Tabatabaei et al., 2009). Factors that affect hatchability include egg fertility and embryonic mortality (Fairchild et al., 2002). Some of the causes of embryonic mortality have been reported to include; prolonged egg storage time, season of the year, nutritional status of hen, egg size, age of breeders and technical incubation problems (Fasenko et al., 2001).

The sperm activities at the site of fertilization in the chicken, especially following cold storage can induce a reduction in the total number of chicks hatched. This is mostly due to the fact that relatively lower numbers of sperms survive the cold storage process satisfactorily, to retain their fertilizing power (Bramwell, 2002). This lower hatchability is associated with a reduction in fertility, as well as an increase in embryonic mortality, which is common in older hens or a flock experiencing infrequent mating or AI activity (Bramwell, 2002). The higher fertility and hatchability recorded in this study from semen extended with EYP+PBS and preserved at 4℃ for 72 hours showed that the extender protected the sperm cells from cold shock. The lowest percentage hatchability after cold storage was recorded in the EYP group. This may be due to lack of antibiotics to protect the viability of the sperm cells because antibiotics increase fertility when included in the semen diluent (Sexton, 1980; Bearden et al., 2004). Furthermore, the general low fertility of the cold preserved semen in this study is consistent with reports that hens inseminated with fresh semen produce more fertile eggs than hens inseminated with cold-stored sperm (Blanco et al., 2000; Long, 2006).

Conclusions

Fertility and hatchability results from this study showed that egg yolk plasma combined with phosphate buffered saline was more effective than whole egg yolk combined with phosphate buffered saline to preserve the fertilizing ability of extended semen for immediate use or storage at 4℃ for up to 72 hours. Therefore, it can be concluded from this study that:

- Cock semen extended in egg yolk plasma alone compares favorably with fresh semen in terms of fertilizing ability, if used immediately after extension. Therefore, it is recommended that egg yolk plasma could be used to increase the volume of semen thereby increase the number of hens that would be inseminated and thus reduce the time and cost spent in AI procedure.
- Cock semen extended with egg yolk plasma in phosphate buffered saline can undergo cold storage for up to 72 hours without impairing egg fertility. Therefore, it is recommended that cold storage (at 4℃) for up to 72 hours of semen with egg yolk plasma plus phosphate buffered saline as extender could be used to keep the viability of the cock semen.
- This study provides additional information on semen extender for AI in poultry. It confirmed that egg yolk plasma can replace whole egg yolk as a component of extender for cock semen.

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