Spermatozoa Survival Rate in Stored Cock Semen Extended With Diluent Fortified With Natural Antioxidant Sources under Room Temperature

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Abstract

An experiment was conducted in a completely randomized design with five treatments in triplicates. Four extenders were formulated: Lake Extender; Coconut water-Orange juice Extender (CWOE); Egg yolk-Tomato juice Extender (EYTE) and Milk based-Carrot juice Extender (MCE), were used to extend cock semen constituting treatments 2, 3, 4 and 5 respectively, while the control (Treatment 1) was unextended semen. Semen was harvested from 8 proven Isa breeder cocks aged 35-40 weeks, pooled and evaluated. The ejaculate was divided into 5 and each part was extended with the extenders at ratio 1:2 (Semen: Extender). The extended and un-extended semen samples were kept under room temperatures (26.6-27.4^{0}C) with relative humidity of 64.0-74.0% for in vitro assessment at 2 hrs interval until percentage motility was less than 60%. The results showed that the mass activity, sperm motility and percentage livability reduced p<0.05) as the time of storage increased. Mass activities of unextended semen and the one extended with coconut water-orange juice were significantly (P<0.05) higher than that of other treatments at 0 hour period of storage. Sperm motility in treatments 1 (95.0%), 3 (93.3%) and 4 (88.3%) was not significantly (p>0.05) different from one another, but they was significantly (p<0.05) higher than treatments 2 (40.0%) and 5 (71.7%). At 2 hours of storage, the mass activity and percentage sperm motility in treatment 2 were significantly (P<0.05) lower than other treatments, while live sperm cells were significantly lower for Treatment 3 than other treatments. At 4 hours of storage, mass activity and motility were zero in Treatment 2, while Treatment 1 recorded the highest values (66.7% and 93.5% respectively). Percent motility in Treatments 3, 4 and 5 were 55.0, 50.0 and 43.3%, respectively. However, percent live sperm cells were significantly (P<0.05) higher in treatments 2, 4, 5 and 1 than the in treatment 3. Both CWOE and EYTE possessed a good keeping quality for semen storage. The extenders fortified with natural antioxidants can be used for on-farm insemination in poultry within two hours of collection.

Keywords: Antioxidant fortified extender, cock semen, spermatozoa survival rate, artificial insemination

Introduction

In poultry, the use of freshly diluted semen (within 20 minutes) proved to be a means of higher fertility (Figueiuredo *et al.*, 1999). It seems impossible to store undiluted cockerel or turkey semen *in vitro* for more than half an hour without losing its fertilizing capacity. An important reason for the decrease in motility and viability during the storage of semen is the formation of lipid peroxides from oxygen radicals (Jones and Mann, 1977). Since poultry semen is very low in volume and highly concentrated, there is the need to develop an appropriate semen extender which is easily accessible and affordable to the poultry breeders.

Diluents and semen storage techniques are

designed to minimize the rate of loss of viability of spermatozoa *in vitro*. Several factors play a role in maintaining the quality of semen over storage. For example, the diluents used for semen extension and storage conditions such as time, aeration, and holding temperature play a major role. It is known that sperm motility and the fertilizing ability of undiluted neat fowl semen stored *in vitro* usually decreases within 1 h of collection (Carter *et al*., 1957). Clarke *et al*. (1982) reported that sperm motility from both undiluted and diluted chicken semen is lowest when stored at 41^0C, which is near the body temperature of the hen. They also revealed that sperm motility was not affected by dilution at storage temperatures of 15 and 5^0C. Therefore, to store fowl semen, type of diluent and storage temperature are very important to avoid a reduction in sperm quality.

Gardener *et al.* (2000) studied the relative contributions of vitamin C, carotenoids and phenolic to the antioxidant potential of fruit juices, including orange, grapefruit, pink grapefruit, apple, pineapple and vegetable-juices and found that both vitamin concentrate-ions and total phenolic contents strongly correlated with antioxidant capacity. This experiment was therefore conducted to determine the sperm cell holding capacity of the different extenders formulated at room temperature. It further examined the effect of antioxidant potential of fruit juices supplemented in the various extenders formulated.

Materials and methods

Experimental site

The experiment was carried out in the laying hen unit of Teaching and Research Farm, University of Ibadan, Ibadan and the extenders were prepared in the Animal Physiology Laboratory in the Department of Animal Science. The extenders were stored inside the freezer throughout the experimental period.

Animal Feeding and Management

In this study, 8 Isa breeder cocks (35-40 weeks of age) were used. Feed and water were administered as recommended by the breeders for birds used for artificial insemination in an open sided pen covered with wire mesh.

Preparation of Poultry Semen Extender

Three different unconventional semen extenders used for this study were prepared based on the existing method of preparation (Lake and Ravie, 1979), which was slightly modified by addition of different natural antioxidants to serve as chemo-protective agents against oxidative rancidity. The gross compositions of extenders are as shown in Table 1.

Data Collection

Prior to the commencement of the experiment, the cocks were trained for semen collection for 2-3 weeks, after which semen was harvested twice a week and ejaculate was taken to the laboratory for *in-vitro* analysis. The semen samples were collected two times a week throughout the 6 weeks of the study by dorsal-abdominal massage method. Care was taken to avoid any contamination of semen with cloaca products such as faeces. Yellow and abnormal semen samples were also discarded to avoid the deleterious seminal products. The ejaculates of 8 cocks were evaluated before use. The semen was pooled and the volume was measured. The pooled semen was split into five treatment groups and diluted with the four various extenders with treatment 1: Unextended semen (control), treatment 2: Lake Extender (conventional extender), treatment 3: Coconut Orange Juice Extender, treatment 4: Egg-yolk Tomato Juice Extender and treatment 5: Milk-

Based Carrot Juice Extender semen. Each treatment was replicated thrice and diluted to insemination doses. Diluted samples were mixed thoroughly and assessment of the diluted and undiluted semen samples was done immediately after dilution. Dilution rate was 1:2, (semen: diluents). Extended semen according to treatments was evaluated for mass activity, motility, concentration and percentage live-dead ratio under phase contrast microscope with appropriate magnification at 0h, 2hrs and 4hrs respectively until percentage motility was less than 60% as described in Ewuola and Egbunike (2010).

Statistical Analysis

The data obtained were subjected to One-way analysis of variance (ANOVA) according to SAS (2003). Differences in mean values were considered significant at the probability level $p<0.05$.

Table 1: Composition of the various poultry extenders for the experiment

Ingredients	Lake (T2)	Coconut water - orange juice (T3)	Egg yolk– tomato juice (T4)	Milk based- carrot Juice (T5)
Sodium glutamate (g)	1.35	-	-	-
Potassium citrate x H20	0.128	-	-	-
Sodium phosphate (% by vol)		-	80	-
Glucose	0.8	-	-	12.5
Distilled water, final vol. (ml)	100.0	-	-	-
Sodium acetate x 4H20	0.08	-	-	-
Coconut water (% by vol)	-	100.0	-	-
Tomato juice (ml)	-	-	10.0	-
Carrot juice (ml)	-	-	-	10.0
Egg yolk (% by vol)	-	-	20.0	-
Glycerol (% by vol)	-	-	-	3.0
Skimmed milk (%)	-	-	-	87.0
Orange juice (ml)	-	10.0	-	-
Buffer (% by vol).	-	-	-	-
Penicillin (mg/ml)	-	1000	1000	1000
Streptomycin (mg/ml)	-	1000	1000	1000

Results

Un-extended and Extended Semen Evaluation under Room Temperature at 0hr (Temp. 26^{0}C and Rel. Humidity: 74%)

The results of un-extended and extended semen evaluation at zero hour of storage under room temperature are shown in Table 2.0. The sperm mass activity was highest in treatments 1 and 3 having the same value of (100%) but significantly different ($P<0.05$) from treatments 2, 4 and 5, with treatment 2 having the lowest value of (38.3%). Sperm motility of treatment 1 was not significantly different from those in treatments 3 and 4, but significantly ($P<0.05$) higher than those from treatments 2 and 5. In the extended semen (T2, T3, T4 and T5) was not significantly different from the un-extended semen (T1). (Recast)

Un-extended and Extended Semen Evaluation under Room Temperature at 2hrs (Temp. 26.6^{0}C; Relative Humidity 72%)

The results obtained when un-extended and extended semen was kept at room temperature (Temp-26^{0}c, RH-76%) for 2hours are shown in Table 3. The mass activity of semen in treatment 3, 4 and 5 was not significantly different from those in the un-extended semen (T1) but they were significantly ($P<0.05$) higher than those in lake extender (T2) which had no mass activity. Percentage sperm motility in

treatments 3 and 4 was not significantly different from the control, but they were significantly ($p<0.05$) higher than those in treatments 2 and 5. Sperm motility was zero in treatment 2 after 2hrs of storage at room temperature. Percentage live sperm cells were highest in treatment 2 (100%), and was statistically similar to treatments 1, 4 and 5 (94.5%, 98.6% and 99.0% respectively), with the exception of treatment 3 which recorded the lowest value ($p<0.05$)...

Un-extended and Extended Semen Evaluation under Room Temperature at 4hrs (Temp=27.4^{0}C; Relative Humidity =64%)

Semen evaluation result at 4hrs of storage at room temperature (Temp-27.4, RH-64%) for extended and un-extended cock semen is presented in Table 4. The mass activity of treatment 1 was the highest, followed by treatment 3, while treatments 4 and 5 had the same value. Treatment 2 had zero mass activity. Motility of treatment 1 was significantly ($P<0.05$) higher than that of treatments 2, 3, 4 and 5... Percentage live sperm in T1, T2, T4 and T5 was similar but significantly ($P<0.05$) higher than that of treatment 3.

Table 2: Un-extended and Extended Semen Evaluation under Room Temperature at 0hr (Temp=26^0, Rel. Humidity 74%)

Parameters	T1 (Unextended)	T2 (Lake)	T3 (COWE)	T4 (EYTE)	T5 (MCE)	SEM
Mass activity (%)	100.0^a	38.3^c	100.0^a	77.8^b	66.7^b	6.87
Motility (%)	95.0^a	40.0^c	93.3^a	88.3^a	71.7^b	5.68
Live sperm cell (%)	100.0^a	100.0^a	100.0^a	100.0^a	100.0^a	0.00

a,b,c; means along the same row with different superscripts are significantly ($P<0.05$) different
SEM: Standard Error of Mean, COWE: Coconut water orange juice extender, EYTE: Egg yolk tomato juice extender, MCE: Milk-based carrot juice extender

Table 3: Un-extended and Extended Semen Evaluation under Room Temperature at 2hrs (Temp =26.6^0, Rel. Humidity 72%)

Parameters	T1 (Unextended)	T2 (Lake)	T3 (COWE)	T4 (EYTE)	T5 (MCE)	SEM
Mass activity (%)	77.8^a	0.0^b	77.8^a	66.7^a	66.7^a	8.27
Motility (%)	81.7^a	0.0^c	81.7^a	73.3^a	66.7^b	8.35
Live sperm cell (%)	94.5^a	100.0^a	54.2^b	98.6^a	99.0^a	4.93

a,b,c; means along the same row with different superscripts are significantly ($P<0.05$) different
SEM: Standard Error of Mean, COWE: Coconut water orange j uice extender, EYTE: Egg yolk tomato juice extender, MCE: Milk based carrot juice extender

Table 4: Un-extended and Extended Semen Evaluation under Room Temperature at 4hrs (Temp. =27.4^{0}c, Relative Humidity 64%)

Parameters	T1 (Unextended)	T2 (Lake)	T3 (COWE)	T4 (EYTE)	T5 (MCE)	SEM
Mass activity (%)	66.7^a	0.0^c	44.4^b	33.3^b	33.3^b	6.10
Motility (%)	70.0^a	0.0^c	55.0^b	50.0^{bc}	43.3^c	6.40
Live sperm cell (%)	93.50^a	66.90^b	53.97^c	97.0^a	97.6^a	6.16

a,b,c; means along the same row with different superscripts are significantly ($P<0.05$) different
SEM: Standard Error of Mean , COWE: Coconut water orange juice extender , EYTE: Egg yolk tomato juice extender, MCE: Milk based carrot juice extender

Un-extended and Extended Semen Evaluation under Room Temperature at 0hr

During the time of storage, a gradual decrease in mass activity, motility and percentage live sperm cells of un-extended and extended semen with various unconventional extenders were observed.

This corroborates the findings of other investigators which reported that during the time of storage, a decrease in live, morphologically normal spermatozoa and an increase in dead and abnormal (bent necks) spermatozoa were observed (Łukaszewicz, 1988; Blesbois *et al.,* 1999). These results suggest that the Lake, CWO, EYT and MCE extenders had varied effect on sperm viability. The Lake extender seemed to have the least beneficial effect on both fresh and stored semen. This confirms the results of earlier studies which demonstrated the comparison between lake extender and two other media, only in lake extender did the number of live spermatozoa in chicken semen stored for 6hrs at 41^0c decline significantly (Howarth, 1979).

At 0hr, mass activity and motility of un-extended and coconut water-orange juice extender were the same. This may be as a result of coconut water containing high energy, nutrients and antioxidants in it, which probably supplied more energy to the sperm cells for their metabolic activities. Also, the contribution of orange juice as an effective antioxidant cannot be under estimated. Also mass activity and sperm motility of egg yolk –tomato juice extended semen and milk-based carrot juice extended semen compared favourably with that of unextended semen which serves as reference control in this study. It was established that sperm contains sperm glycocalyx (Bearer and Friend, 1990) which has been reported to be a dense carbohydrate layer extending 20-60mm from cell surface that emanates from either plasma membrane proteins (glycoproteins) or lipids (glycolipids) and represent the primary interface between sperm cells and its environment. This corroborated the findings of Clarke *et al*. (1982) who reported that dilution of chicken semen increases sperm motility. Percentage live sperm cells were similar across the treatments.

Un-extended and Extended Semen Evaluation under Room Temperature at 2hrs (Temperature: 26.6^0, Relative Humidity: 72%)

At 2hrs of storage under room temperature of 26^0c and relative humidity of 74%, there was a gradual reduction in the mass activity, motility and percentage live sperm cells in extended and unextended semen. This corroborated the results of previous studies by Lukasewicz (1988) and Blesbios *et al*. (1999) who observed that during the time of storage, a decrease in live, morphologically normal spermatozoa and an increase in dead spermatozoa and spermatozoa with bent necks were observed. This may be attributed to the fact that synthetic substances or chemicals do not have ability to maintain sperm motility and mass activity *in vitro* under room temperature for a long time. Percentage live sperm cells of Lake Extender were highest but similar to other treatments, with the exception of CWOE. This is an indication that coconut water orange juice extender does not have the ability to keep sperm cells alive for a long time probably because the energy might have been loss, while Lake Extender is capable of keeping the cells alive but immobilized. According to Howarth (1979), the Lake extender seemed to have the least beneficial effect on both fresh and stored semen. This agreed with the results of earlier studies which demonstrated the comparison between Lake Extender and two other media; only in Lake Extender that the number of live spermatozoa in chicken semen stored for 6hrs at 41^0c decline significantly. Also turkey spermatozoa rapidly lost viability and fertilizing capacity when stored either undiluted or diluted at physiological temperatures (Leighton *et al*.,

1968; Lake and Ravie, 1982).

Un-extended and Extended Semen Evaluation under Room Temperature at 4hrs (Temperature-27.4^{0}c, Relative Humidity-64%)

At 4hrs of storage under room temperature, mass activity, motility, percentage live sperm cells still gradually declined compared to when at 0 and 2hours of storage across the treatments. Both extended and un-extended semen were affected, however semen still had a fairly comparable value to other treatments. Clarke *et al*. (1982) reported a significant reduction in the percentage of progressively motile chicken and turkey spermatozoa when compared to fresh semen or semen samples held at lower temperatures such as 5, 15, or 25^{0}C.

The CWOE and EYTE were observed to be the best extenders that have the highest keeping value among the four extenders used in this study. The wholesome effects of the extender fortified with orange juice can be attributed to it excellent source of vitamin C (Mermeistein, 1999; Martin *et al*., 2002), which acted as water–soluble antioxidant to scavenge aqueous peroxide radicals before these destructive substances could have a chance to damage the lipids (Wainer *et al.,* 1986). Also the lycopene content of tomato may also be the major contributor to the storage shelf life of the sperm cells in tomato juice fortified extender (ETTE). Lycopene possesses a singlet-oxygen-quenching ability 10 times higher than that of α-tocopherol **(**Di Mascio and Sies, 1989**).** The α-tocopherol has been demonstrated to reduce the susceptibility to lipid peroxidation and improved semen quality traits **(**Brezezinska *et al*., 1995**;** Geva *et al*., 1996**;** Kessopoulou ***et al.,* 1995).** Therefore, the use of tomato juice as a lycopene-rich source in the semen diluent in this study may be the probable reason for the keeping quality of the extender at the room temperature for up to 4hrs.

Conclusion and Application

The results of this experiment revealed that both coconut water orange juice extender and egg yolk- tomato juice extender possessed a good keeping quality for semen storage. Therefore, the two extenders could be used to extend semen for cold storage for the assessment of their keeping quality. Besides, the extenders fortified with natural antioxidants can be used for on-farm artificial insemination within two hours of collection.

References

Bearer, E. L. and Friend. D. S. 1990. Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. *J. Electron Microsc. Tech.* 16:281–297.

Blesbois, E., Grasseau, I. and Hermier. D. 1999. Changes in lipid content of fowl spermatozoa after liquid storage at 2 to 5°C. *Theriogenology* 52:325–334.

Brezezinska-Slebozinska, E. A., Slebodzinki, B., Pietras, B. and Wieczorek, G. 1995. Antioxidant effects of vitamin E and glutathione on peroxidation in boar semen plasma, *Bio. Tra. Ele. Res.,* 47, 69-74.

Carter, R. D., McCartney, M. G., Chamberlin, V. D. and Wyne, J. W. 1957. The effect of storage time and temperature on fertilizing capacity of turkey semen. *Poult. Sci*., 36: 618-621.

Clarke, R. N., Sexton, T. J. and Ottinger, M. A. 1982. Effect of holding temperature and storage time on respiratory rate, motility, and

fertility of chicken and turkey semen. *Poult. Sci*., 61: 1912-1917.

DiMascio, P., Kaiser, S. and Sies, H. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher, *Arch. Biochem. Biophy*s., 274, 1989, 532-538 disease. *Hum. Reprod. Update*, 10, 149-162.

Ewuola, E. O. and Egbunike, G. N. 2010.

Figueiuredo, E. A. P., Souse, F. M. D, Guidoni, A. L. and Rosa, P. S. 1999. Effects of composition of diluents, dilutions and storage time of heavy broiler breeder semen on fertility, hatchability and chick production. *Revista Brusileria de Zootecnia*, 25: 1239-1244.

Gardner, P. T., Tamsin, T. C., White, A. C., Mcphail, D. B. and Duthie, G. P. 2000. The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. *Food Chemistry,* 68: 471-474.

Geva, E, Lessing, J. B., Bartoov, B., Lenergeva, L., Zabludovsky, N. and Amit, A. 1996. The effect of antioxidant treatment on human spermatozoa and fertilization rate in an *in vitro* fertilization program, *Fert. Ster*., 66, 430-434.

Howarth, B., Jr. 1979. Comparison of extenders for holding cock semen six hours at 41°C. J. *Reprod. Fertil.* 57:149–155.

Jones, R. and Mann T. 1977. Damage to ram spermatozoa by peroxidation of endogenous phospholipids. *Journal of Reproduction and Fertility,* 50: 261-268.

Kessopoulou, E., Powers, H. J., Sharma, K. K., Russel, M. J., Cooke, I. D. and Barratt, C. L. R. 1995. A double-blind randomized placebo crossover control trial using the antioxidant vitamin E to treat reactive oxygen species associated male fertility. Fertile. Steril. 64: 825-831.

Lake, P. E. and Ravie, O. 1979. Effect on fertility of storing fowl semen for 24 h at 58C in fluids of different pH. J. *Reprod. Fertil.* 57, 149–155.

Leighton, A, Carte, I., Potter, I., Coleman, J. and Beane, W. 1968 The effects of storage temperature, storage time and dietary vitamin E on fertilizing capacity and physiological

Łukaszewicz, E. 1988. Study of extenders for cock's semen storage in the light of laboratory estimation and fertility rates (in Polish). Zeszt. Nauk. AR we Wrocławiu, 168:43–59.

Mermeistein, N. H. 1999. High–pressure pasteurization of juice. *Food Technology,* 53: 86-90.

SAS. 2003. Statistical Analysis System . SAS stat. Version 9. SAS Institute Inc. Gary NC U.S.A.

Wainer, D. D. M., Burton, G. W. and Ingold, K. U. 1986. The antioxidant efficiency of vitamin C is concentration-dependent. *Biochimica et Biophysica Acta,* 884: 119-123.

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