IN VITRO EFFECTS OF SELENIUM AND α-TOCOPHEROL INCLUSION ON SPERMATOZOA QUALITY OF EXTENDED COCK SEMEN STORED UNDER ROOM TEMPERATURE

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ABSTRACT

Cock spermatozoa are highly susceptible to deterioration during storage *in vitro,* hence, the need to study the effect of α-tocopherol and selenium in Ringer's solution as cock semen extender. Semen samples were collected from fifteen Lohmann breeder cocks, pooled and divided into nine groups: Ringers Solution (RS) without antioxidant (T1), RS with α-tocopherol at 25 (T2), 50 (T3), 75 (T4) or 100µgm/l (T5) and selenium at 25 (T6), 50 (T7), 75 (T8) or 100µgm/l (T9). All treatments were stored under room temperature (27 – 29 ^{o}C) and evaluated every 3hours until motility dropped below 50%. Spermatozoa Motility (SM), Liveability, Total Antioxidant Capacity (TAC, mmol/l) and Lipid Peroxidation (LP, µMMDA/l0^6 Spermatozoa) were determined using standard procedures. Data were analysed using ANOVA at $\alpha_{0.05}$. Result showed that progressive motility decreased below 50% after 6 hours of storage. α-tocopherol inclusion level at 100µgm/L for SM (91.7%) and liveability (94.5%) at 3h evaluation period were significantly higher than control (81.7%) and T9 (85.2%), respectively. The TAC ranged from 2.0 (T9) to 3.2 (T5), while LP ranged from 0.5 (T1) to 2.8 (T7) at 3 hours. It was concluded that α-tocopherol inclusion at 100µgm/l in Ringers solution improved cock spermatozoa motility and liveability.

Keywords: Spermatozoa, motility, selenium, semen extension, ringer's solution

INTRODUCTION

The poultry industry has the most viable market of all the agricultural industry in Nigeria. Among the poultry birds which are commonly raised in Nigeria are ducks, turkeys, chickens, pigeons, ostriches, and guinea fowls. The most commercialized or economically viable amidst them includes turkeys; guinea fowls and chicken while the most predominant amongst them is chicken (Adene and Oguntade, 2006).

Artificial insemination (AI) is increasingly used in sheep, beef cattle, horses as well as poultry and has been adopted for other domestic species such as buffalo, deer, goats and dogs. It has also been used sporadically in breeding and preservation of endangered or rare species (Morrell, 2011). Poultry farmers in the years past had used genetic selection and enhanced feeding management to increase growth rate in poultry production which in turn had certain detrimental effects on reproduction (Bramwell, 2002). Artificial Insemination (AI) in poultry is going to be more familiar (in solving the problem of breeding) to the poultry farmers as well as poor villagers due to its practical impact from economical point of view. It is true that its practicability is still far from small holder investors due to lack of available relevant technologies, but with the introduction of simple equipment and procedures, the benefit can be realized (Yahaya, Umaru and Aliyu; 2013). The merits of AI includes improved biosecurity, reduced stress on females, fewer number of males required, enhanced semen preservation and increased mating ratio

Semen preservation with the use of the right diluents is an essential factor in the success of artificial insemination in animal reproduction. According to Bilodeau, Blanchette, Gagnon and Sirard (2001), dilution of semen by a diluent leads to a low level of antioxidants which may partly explain the lower fertility rate caused by high level of lipid peroxides production which is common to poultry semen. Several conventional diluents including ringer's solution do not have antioxidants as part of their constituent. This research therefore seeks to test the implication of selenium and α-tocopherol in ringer's solution on Lohmann breeder cock spermatozoa quality.

MATERIALS AND METHODS

Experimental animals

Fifteen (15) 30-weeks old Lohmann breeder cocks were used for this study. All routine and occasional management practices were carried out as at when due. The birds were housed individually in battery cages and provided with clean water and fed *ad libitum* throughout the study.

Extender Preparation

The extender used for the study was Ringer's solution and its composition is shown in Table 1. The solution was neutralised (pH 7.0) by adding 2 drops of diluted acid (10M of H_2SO_4) to the extender and kept in the refrigerator until when needed.

The test ingredients (α-tocopherol and selenium) were obtained from Hi-Nutrients International Limited, Lagos, Nigeria. They were powdery substances in the form of vitamin ED_{50} and sodium selenite; vitamin D and sodium acting as carrier for the tocopherol and selenium respectively.

Table 1: The chemical composition of the Ringer's solution

Constituents	Grams/Litre
Sodium Chloride	6.80
Potassium Chloride	1.73
Calcium Chloride	0.64
Magnesium Sulphate	0.25
Sodium Bicarbonate	2.45
D H_2O added to the mixture to reach 1000 ml	

Source: Tabatabaei Batavani and Ayen(2011)

Semen Collection and Evaluation

Semen was harvested from 15 cocks by using the abdominal massage semen collection method (Burrows and Quinn, 1935). The semen collected into an ependorf tubes was evaluated for colour and impurities after which it was pooled together, maintained at 37 ^oC and taken to the laboratory for extension and qualitative evaluation.

The volume of the pooled semen was determined and divided into aliquots of ratio 1:10 semen to extender according to treatments with 3 replicates in a completely randomized design.

Experimental treatment allotment
Treatment 1: Semen + RS (Control)
Treatment 2: Semen + RS with 25 µg m/l of α-Tocopherol
Treatment 3: Semen + RS with 50 µg m/lof α-Tocopherol
Treatment 4: Semen + RS with 75 µg m/l of α-Tocopherol
Treatment 5: Semen + RS with 100 µg m/l of α-Tocopherol
Treatment 6: Semen + RS with 25 µg m/lof Selenium
Treatment 7: Semen + RS with 50 µg m/l of Selenium
Treatment 8: Semen + RS with 75 µg m/l of Selenium
Treatment 9: Semen + RS with 100 µg m/l of Selenium

* RS= Ringer's Solution

The extended semen was then stored inside at room temperature (27-29 ^oC) and evaluated every 3 hours for progressive motility, percentage liveability, total antioxidant capacity and lipid peroxidation.

Sperm Motility: Sperm motility was determined by putting a drop of freshly collected semen with a drop of sodium citrate and was placed on glass slide on a warm stage at 37 °C. Observations were made at × 400 magnifications on the sample under microscope to determine sperm motility (Hafez and Hafez, 2000).

Percentage Livability: This was done by placing a drop of semen on a warm glass slide, one drop of Eosin-Nigrosin stain was added and mixed gently, it was then smeared on a slide with the edge of another clean slide, air dried and viewed under the microscope at magnification of ×400. The dead sperm cells absorbed the stain while live sperm cells did not. The live sperm cells were counted and placed as a fraction of the total number of sperm cells that were present (Ewuola and Egbunike, 2010).

Lipid Peroxidation**:** 3ml each of glacial acid and 1% TBA solutions were added to test tubes appropriately labeled blank and tests following the procedure by Mokogwu, Airhomwanbor, Mokogwu and Onohwakpor (2016). 0.6ml of distilled water was added to the blank, while 0.6ml of treatment serum was added to each of the test tubes. These were thoroughly mixed, incubated in a boiling water bath for 15minutes, then allowed to cool, after which they centrifuged and their supernatants collected. The supernatant from the blank was used to zero the spectrophotometer (preset at 532nm) before reading the absorbance of the supernatants from the test solutions. The concentration of Malanoaldehyde in the serum was then calculated as follows

$$\frac{\text{Absorbance of test at 532nm} \times \text{total volume of the reaction mixture} \times 1000}{(56 \times 10^5 \text{m}^{-1}\text{cm}^{-1}) \times \text{volumeof semen} \times 1\text{cm}}$$

Total Antioxidant Capacity was done according to procedure of Koracevic, Koraceivic, Djordjevic, Andrejevic and Cosic(2001)

Statistical Analysis

Data obtained were analysed using the analysis of variance procedure of SAS (2003) and means were separated using the Duncan's multiple range test of the same software.

RESULTS

Effects of α-tocopherol and selenium inclusion on spermatozoa progressive motility of extended cock semen stored at27 to 29 °C

The results of the effects of α-tocopherol and selenium fortified extender on progressive motility of stored semen (27 to 29 °C) are shown in Table 2. There was no significant difference (P>0.05) except at 3 and 9 hours post storage. At 0 hour, values obtained range from 91.67% in 25 µgm/l of α-tocopherol (T2), 75 µgm/l and 100µgm/lof selenium (T8 and T9respectively) to 96.33% in 75 µgm/lof α-tocopherol(T4). At 3 hours, 100 µgm/l of α-tocopherol (T5: 91.67%) was significantly (P<0.05) higher than control (T1: 81.67%) and 50 µgm/l of selenium inclusion level (T7: 78.33%) but similar to other treatments. However, 50 µgm/l of α-tocopherol inclusion level (T3: 88.33%) was also statistically different (P<0.05) from 50 µgm/l of selenium inclusion T7 (78.33%). The values obtained at 6 hours ranged from 63.33% in T1 and T4 (control and 75 µgm/l of α-tocopherol, respectively) to 73.33% in T2 (25 µgm/l of α-tocopherol). At 9 hours post storage, all treatments were significantly similar (P>0.05) except for 100 µgm/l of α-tocopherol inclusion level (T5: 16.67%) and 50µgm/l of selenium inclusion level (T7: 15.00%) which were significantly higher (P<0.05) than control (T1: 1.67%). Mean values lower than 50% were recorded after 6 hours post storage indicating that good extended cock semen motility stored at room temperature of 27-29 °C cannot exceed 6 hours.

Table 2: Effects of α-tocopherol and selenium fortified extender on progressive spermatozoa motility (%) at room temperature

Storage period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	

0 hour	93.33	91.67	95.00	96.33	93.33	95.00	94.00	91.67	91.67	0.83
3 hours	81.67^{bc}	83.33^{abc}	88.33^{ab}	85.00^{abc}	91.67^{a}	86.67^{abc}	78.33^{c}	83.33^{abc}	85.00^{abc}	1.60
6 hours	63.33	73.33	66.67	63.33	71.67	68.33	65.00	70.00	68.33	2.63
9 hours	8.33^{ab}	11.67^{ab}	6.67^{ab}	13.33^{ab}	16.67^{a}	1.67^{b}	15.00^{a}	10.00^{ab}	13.33^{ab}	2.10

a, b, c: Means along the same row with different superscript are significantly (P<0.05) different.

Effects of selenium and α-tocopherol inclusion on spermatozoa percentage liveability of extended cock semen stored at27 to 29 °C

The results of the effects of α-tocopherol and selenium fortified extender on percentage liveability of stored semen (27 to 29 °C) are shown in Table 3. Significant differences (P<0.05) were observed only at 3 and 6 hours post storage. At 0 hour, mean values obtained range from 93.33% in 25µgm/l of α-tocopherol level(T2) to 99.17% in 75 µgm/l of α-tocopherol inclusion level (T4). At 3 hours, all mean values were statistically similar (P<0.05) except for T5:100 µgm/l of α-tocopherol inclusion level (94.50%) which was significantly higher (P<0.05) than T1: control (85.17%), T2: 25µgm/l of α-tocopherol inclusion level (84.67%) and T9: 100µgm/l of selenium inclusion level (83.17%). At 6 hours, all values obtained were not significantly different (P>0.05) except for 25 µgm/l of α-tocopherol inclusion level (T2: 89.00%) which was significantly higher (P<0.05) than 75 µgm/l of selenium inclusion level (T8: 71.83%). Mean values obtained at 9 hours post storage ranged from 45.33% in T1 (control) to 72.33% in T7 50 µgm/l of selenium inclusion level. Thus, percentage liveability of extended cock spermatozoa can be preserved under room temperature (27 to 29 °C) if supplemented with α-tocopherol and selenium.

Table 3: Effects of α-tocopherol and selenium fortified extender on spermatozoa liveability (%) at room temperature

Storage period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
0 hour	95.33	93.33	93.50	99.17	95.83	97.00	96.83	96.67	95.00	1.16
3 hours	85.17^{b}	84.67^{b}	89.67^{ab}	90.17^{ab}	94.50^{a}	91.50^{ab}	91.67^{ab}	88.33^{ab}	83.17^{b}	1.55
6 hours	73.50^{ab}	89.00^{a}	86.50^{ab}	86.00^{ab}	81.17^{ab}	72.83^{ab}	82.67^{ab}	71.83^{b}	87.50^{ab}	2.77
9 hours	45.33	48.67	59.33	68.00	59.67	64.00	72.33	49.00	57.67	5.34

a, b, c: Means along the same row with different superscript are significantly (P<0.05) different.

Effects of selenium and α-tocopherol inclusion on total antioxidant capacity of extended cock semen stored at27 to 29 °C

The results of the effects of α-tocopherol and selenium fortified extender on total antioxidant capacity of stored semen (27 to 29 °C) are shown in Table 4. There was significant difference among the treatments at 0 hour evaluation period. Significantly higher (P<0.05) values were observed at T3 (2.71 mmol/l), T5 (2.47 mmol/l),

T8 (2.77 mmol/l) and T9 (2.63 mmol/l), although not different (P>0.05) from T1, T4, T6, and T7, they differ significantly (P<0.05) from T2 (1.06 mmol/l). At 3 hours post storage, mean values obtained range between 2.04 mmol/l in T9 and 3.22 mmol/l in T5. Also at 9 hour, the highest value was observed in T7 (2.49 mmol/l) while the lowest was observed in T2 (1.73 mmol/l). Therefore, the effect of the fortification of extended cock spermatozoa with α-tocopherol and selenium could not be sustained during storage at room temperature (27 to 29 °C).

Table 4: Effects of α-tocopherol and selenium fortified extender on total antioxidant capacity (mmol/l) at room temperature

Storage period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
0 hour	1.96^{ab}	1.06^{b}	2.71^{a}	1.84^{ab}	2.47^{a}	1.80^{ab}	1.78^{ab}	2.77^{a}	2.63^{a}	0.22
3 hours	2.18	2.25	2.35	2.89	3.22	2.67	2.58	2.96	2.04	0.25
6 hours	1.94	1.73	2.07	1.88	2.07	2.07	2.49	2.37	2.05	0.33

a, b, c: Means along the same row with different superscript are significantly (P<0.05) different.

Effects of selenium and α-Tocopherol Inclusion on Lipid Peroxidation of Extended Cock Semen stored at27 to 29 °C

The results of the effects of α-tocopherol and selenium fortified extender on lipid peroxidation of stored semen (27 to 29 °C) are shown in Table 5. There were significant differences among treatments at 0 and 6 hours post storage. At 0 hour, 100 µgm/l of selenium inclusion (T9: 3.80 µM MDA/l0^{-6} Spermatozoa) was significantly higher (P<0.05) than all other treatments. However, values obtained were between 0.46 µM MDA/l0^{-6} Spermatozoa in 100µgm/l of α-tocopherol inclusion level (T5) and 3.80 µM MDA/l0^{-6} Spermatozoa in 100µgm/L of selenium inclusion level (T9). At 3 hours post storage, mean values were not significantly different (P>0.05) among treatments. They ranged from 0.46 µM MDA/l0^{-6} Spermatozoa in control (T1) to 2.75 µM MDA/l0^{-6} Spermatozoa in 50µgm/l of selenium inclusion level (T7). The 2.23 µM MDA/l0^{-6} Spermatozoa (T9: 100 µgm/l of selenium inclusion) at 6 hours post storage was significantly higher (P<0.05) than T2, T3, T4, T5 and T6 (25, 50, 75,100 µgm/L of α-tocopherol inclusion and 25µgm/l of selenium inclusion respectively) but statistically similar (P>0.05) to T1, T7 and T8 (control, 50 and 75 µgm/l of selenium inclusion level).

Table 5: Effects of α-tocopherol and selenium fortified extender on lipid peroxidation (µM MDA/l0^{-6} spermatozoa) at room temperature

Storage period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
0 hour	0.98^{b}	1.38^{b}	0.52^{b}	0.98^{b}	0.46^{b}	1.18^{b}	0.98^{b}	1.38^{b}	3.80^{a}	3.68
3 hours	0.46	0.59	1.38	2.03	1.38	0.79	2.75	2.03	1.51	4.98
6 hours	1.44^{abcd}	0.52^{d}	0.46^{d}	0.69^{d}	0.92^{bcd}	0.72^{cd}	1.90^{ab}	1.83^{abc}	2.23^{a}	1.96

a, b, c: Means along the same row with different superscript are significantly (P<0.05) different.

DISCUSSION

Progressive motility values were reduced close to 0% at 9 hours post storage. Lack of energy source in the extender and the high metabolic activities of the spermatozoa under room temperature (27 to 29 ^{o}C) could be responsible for the rapid decrease below 60% across the treatments after 6 hours of incubation. This could also mean that the fortified extender did not contain the ingredients that could perfectly simulate the *in vivo* milleau of the spermatozoa. This is similar to the work of Tsukunage (1971) and Maldjian, Cerolini, Surai, and Speake (1998) who reported a decline in spermatozoa motility after 6 hours of storage at 25 ^{o}C and Oloye Oyeyemi, Ajala and Akusu (2008) who recommended maximum of 6hrs storage period for buck (post diluted) semen at room temperature. According to Surai, Brillard, Speake, Blesbois, Seigneurin and Sparks (2000), the exposure of seminal plasma to elevated temperatures decreased its protective effect by 50% (where motility is an important indicator) which reflects the importance of antioxidants enzymes Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in antioxidant activity of the plasma. The endogenous adenosine triphosphate content of the mitochondria which supplies energy for motility is depleted during storage and the mitochondria membrane potential is very important in spermatozoa penetration into oocytes. The mitochondria is said to contribute to the production of ROS through nicotinamide adenine dinucleotide (NADH)-dependent oxido-reductase system (Hallak, Sharma, Pasqualotto, Ranganathan Jr, Thomas and Agarwal; 2001) thereby causing reduced motility. At 3 hours post incubation, increasing level of α-tocopherol improved motility. This could be attributed to increased deposition of α-tocopherol on spermatozoa cell membranes. Blesbois, Grasseau and Blum (1993) justified that the efficiency of added α-tocopherol will depend on the extent to which α-tocopherol accumulates in the cell membranes. α-tocopherol was reported by Tabatabaei *et al.* (2011) to bond with endoperoxides to also preserve spermatozoa during storage. The low values obtained at 9 hours post storage in this experiment could be attributed to increased metabolic activities resulting in energy depletion of endogenous nutrients and thus will not be recommended for insemination.

Percentage live cells at 3 hours showed that increasing level of α-tocopherol inclusion improved spermatozoa. The observed improvement on spermatozoa liveability when α-tocopherol level was increased agreed with the report of Surai and Ionov (1992) and Donoghue (1997) who stated that α-tocopherol improved semen viability during storage in turkey when incorporated into an extender but the reverse was the case with selenium-based treatments. Barber, Parker and Mcdaniel (2005) demonstrated that *in vitro* addition of selenium to fowl semen at very high dose may be detrimental to spermatozoa in terms of semen quality index. Barber *et al.* (2005) explained that the mineral may indirectly improve the semen quality during spermatogenesis rather than acting directly on spermatozoa. This may also be related to the form of selenium used. α-tocopherol being a lipid soluble antioxidant has been reported by Aitken, Clarkson, Hargreave, Irvine and Wu (1989) to have the ability to penetrate plasma membranes and suppress free radicals thereby preserving spermatozoa liveability. Selenium on the other hand being water soluble is effective by inducing glutathione peroxidase which is an enzyme whose activity is more inside the plasmalemma than in the seminal fluid surrounding the cell. Also, Mahan (1999) reported the assimilation of selenium from organic sources to be much more efficient compared to the commonly used selenite. Unlike in 3 hours storage period, increased α-tocopherol level did not improve spermatozoa liveability at 6 hours post storage. This could probably be due to the inefficiency of the plasma membrane integrity arising from senescence under storage conditions which permits increased metabolic activities *in vitro*. It thus suggests the possibility of impairment to the removal of damaged molecules from the spermatozoa cell membranes.

The increasing level of antioxidant fortification reflected on the total antioxidant capacity test at 0 hour post storage although they did not significantly differ from the control. The values obtained exceeded the range (0.25±0.07) given by Blesbois *et al.* (1993) in washed spermatozoa to 1.6µg/ml in fresh ejaculates which confirmed that antioxidants are naturally present in fresh semen. It was observed in some of the treatments that total antioxidant capacity value increased at 3 hours post storage and later decreased at six hours. This suggests that the release of the antioxidant from the binder into the seminal fluid was gradual with respect to time and the metabolic activities of the spermatozoa cells. At 6 hours post storage, the values for selenium-based treatments were generally higher than that of α-tocopherol which probably suggests that α-tocopherol was more utilized in peroxidative defense compared to selenium. However, it cannot be concluded because their mode of activity against peroxidative damage differs.

It was observed that the presence of exogenous antioxidant did not directly reduce the rate of peroxidation at 0 hour and 3 hours post storage under room temperature (27 to 29 ^{o}C). This could be due to the complexity of antioxidant processes involved in reducing peroxidation *in vitro*. It therefore suggested that the effect of antioxidant defense against peroxidative damage was more of *in vivo* than *in vitro*. This corroborated the work of Long and Kramer (2003) who reported that α-tocopherol did not reduce lipid peroxidation during storage.

The high value of peroxidation at 100µg/ml inclusion level of selenium could be due to agglutination of spermatozoa cells in the evaluated semen sample since the membranes of the cells are made of phospholipids thereby leading to the high value observed even at 0 hour. At 6 hours post storage, lower values of lipid peroxidation were observed and compared to selenium-based treatments and control which further confirmed that α-tocopherol had better potential to reduce the rate of peroxidative damage in extended semen sample under room temperature.

CONCLUSION

Addition of α-tocopherol up to 100µgm/l in Ringer's solution increased progressive motility and percentage liveability of spermatozoa under room temperature which justified the need for its adoption in the poultry industry. However, Selenium and α-tocopherol inclusion in Ringer's solution at different inclusion levels had varied effects on total antioxidant capacity and mitigation of lipid peroxidation during *in vitro* storage.

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