IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 13, Issue 1 Ser. I (January 2020), PP 42-49
www.iosrjournals.org

Combined Effects of Selenium and α-Tocopherol on Quality and Fertilising Ability of Spermatozoa in Extended Cock Semen Processed and Inseminated On-Farm

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Abstract: *Spermatozoon apoptosis in extended semen is a challenge to artificial insemination in poultry production. Extender supplementation with exogenous antioxidants could mitigate lipid peroxidation and improve semen quality. Therefore, effects of selenium and α-tocopherol on quality and fertilising ability of spermatozoa in extended cock semen were assessed. Semen samples were collected from fifteen Lohmann breeder cocks aged 40 weeks, pooled and divided into five groups. Each group was extended with Ringers Solution (RS) without antioxidant (T1), RS+25µgmL^{-1} α-tocopherol (T2), RS+25µgmL^{-1} selenium (T3), RS+12.5µgmL^{-1} α-tocopherol+12.5µgmL^{-1} selenium (T4), RS+25µgmL^{-1} selenium+25µgmL^{-1} α-tocopherol (T5) were assessed in vitro and in vivo. All treatments were evaluated for Spermatozoa Progressive Motility (SPM), Spermatozoa Liveability (SL), Total Antioxidant Capacity (TAC, mmol/L) and Lipid Peroxidation (LP, µMMDA/l0^{6} Spermatozoa) were determined using standard procedures. Seventy-five Lohmann breeder hens aged 40 weeks were divided into five groups and inseminated with each of the treatments under room temperature for the in vivo evaluation. Egg fertility and hatchability were recorded weekly for four weeks. Data were analysed using descriptive statistics, correlation and ANOVA at $\alpha_{0.05}$. Result showed that the effect of α-tocopherol and selenium combinations were not ($p>0.05$) different among treatments for SPM, SL and LP up to 6h. Egg fertility was ($p<0.05$) higher at T1 (86.5±7.3%), T2 (86.0±7.6%) and T3 (82.0±5.0%) than T4 (57.0±7.1%) and T5 (72.3±8.7%), while hatchability of set eggs ranged between 40.0±8.8% (T2) and 71.3±10.2% (T3). Fertility positively correlated with hatchability (r=0.95). Selenium inclusion at 25µgmL^{-1} under room temperature improved hatchability in Lohmann breeder hens.*

Additional Key words: *Fertility, Hatchability, Lohmann breeder cocks, Spermatozoa quality*

Date of Submission: 26-12-2019 Date of Acceptance: 11-01-2020

I. Introduction

Poultry semen has been reported to be highly susceptible to rapid deterioration due to its high susceptibility to lipid peroxidation during storage and processing for artificial insemination (AI) (Surai et al 2000). Earlier studies on chicken and turkey discovered that lipid peroxidation of spermatozoa membranes takes place in just a few hours during *in vitro* storage both at body-like temperatures (37 °C) as well as at 0 °C (Bakst 1993).

In poultry reproduction, it is well established that artificial insemination in avian species has relative advantages as compared with natural mating (Penfold *et al* 2000, Brillard 2003). The susceptibility of poultry spermatozoa to lipid peroxidation has therefore restricted the adoption of stored poultry semen to natural mating in most part of the world as against semen of other livestock such as cattle and swine which can be extended in the laboratory and transported to farm at different locations. According to Agarwal et al (2003), poor spermatozoa quality has been reported to be associated with high reactive oxygen species (ROS) production as consequence of lipid peroxidation in semen. Reactive oxygen species may have useful or damaging effects on spermatozoa depending on the location, time of exposure, concentration and type (Agarwal and Saleh 2002).

The increasing demand for AI in the poultry industry emphasizes the need for the distribution of good quality sperm. In order for the poultry industry to take advantage of modern AI techniques, proper storage of poultry semen is necessary. As chicken semen is highly concentrated and is of low volume, the extension of neat semen with proper diluents is required prior to AI and storage. Ringer's solution has been well established to be useful in culturing biological cells where spermatozoa are no exception. However, most conventional semen extender such as Ringer's solution does not contain antioxidants in their composition.

Antioxidants are the agents, which break the oxidative chain reaction, thereby, reducing the oxidative stress (Kumar and Mahmood 2001). Traber and Atkinson (2007) reported that Tocopherol (α-tocopherol) scavenges peroxyl radical. It functions by maintaining the integrity of long chain polyunsaturated fatty acids in

the cell wall and thereby sustains their biological activity. Selenium is also an essential component of glutathione peroxidase (GSH-PX) and it has been reported as an enzyme that shields cellular components from free radicals and also serves as an antioxidant for cell membrane lipids (Sanchez-Gutierrez et al 2008).

This study therefore investigated the combined effects of selenium and α-tocopherol in extended cock semen on quality and fertilising ability of spermatozoa to provide an enzymatic and biochemical defense mechanism for spermatozoa.

II. Materials And Methods

The study was conducted at the Teaching and Research Farm of the University of Ibadan ($7^{o}25^{'}38.952^{''}$N, $3853^{'}0.63^{''}$E; 200m above sea level). All laboratory analyses were carried out at the Animal Physiology Laboratory of the Department of Animal Science, University of Ibadan, Nigeria.

Experimental Animals

Fifteen (15) Lohmann breeder cocks and seventy five (75) Lohmann breeder hens were used in this study. The cocks and hens were 40 weeks old. They were provided feed and water *ad libitum* throughout the experimental period. The cocks were housed individually in battery cages while the hens were three per cubicle of a battery cage with measurement 20 X 17 X 16 inches which represents height, length and width respectively). All the cages were kept clean and routine management practices were carried out regularly.

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 (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

Distilled H_2O was added to the mixture to make up 1000 mL

Source: Tabatabaei et al (2011)

Semen Collection and Evaluation

Semen was harvested from 15 cocks by using the abdominal massage semen collection method. The semen collected into an eppendorf tubes were first screened on site for colour and impurities before pooling together and maintained at 37 ^{o}C. It was later transferred to the laboratory for extension and qualitative evaluation.

The volume of the pooled semen was determined and divided into aliquots of ratio 1:5 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 25µgmL^{-1} of α-tocopherol
Treatment 3: Semen + RS with 25µgmL^{-1} of selenium
Treatment 4: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium
Treatment 5: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium
* RS= Ringer's Solution

The extended semen was monitored every 3 hours for Progressive motility, Percentage livability, Total antioxidant capacity and Lipid peroxidation.

Sperm Motility: Sperm motility was determined by placing a drop (with micropipette) of freshly collected semen with a drop of sodium citrate on glass slide warmed at 37 ^{o}C. Observations were made at × 400 magnifications on the sample under microscope to determine sperm motility (Hafez and Hafez 2000).

Percentage Liveability: 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. Dead sperm cells absorbed the stain while

live spermatozoa remained unaffected. The live sperm cells were counted and placed as a fraction of the total number of sperm cells that are present (Ewuola and Egbunike 2010).

***Lipid Peroxidation*:** Three mL each of glacial acid and 1% thiobarbituric acid (TBA) solutions were added to test tubes appropriately labeled blank and tests following the procedure by Mokogwu et al (2016). Distilled water (0.6 mL) was added to the blank, while 0.6 mL 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 were centrifuged at 2000rpm for 5 mins 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 Malondialdehyde in the serum was then calculated as follows:

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

Total Antioxidant Capacity was done according to procedure of Koracevic et al (2001) by centrifuging the treatments when ready for analysis at 3000rpm for 5 minutes to decant the serum from the sperm cells. A standardised solution of Fe–EDTA complex reacts with hydrogen peroxide by a Fenton-type reaction, leading to the formation of hydroxyl radicals (OH). These reactive oxygen species degrade benzoate, resulting in the release of thiobarbituric acid reactive substances (TBARS). Antioxidants from the added seminal fluid sample causes suppression of the production of TBARS. This reaction was then measured spectrophotometrically and the inhibition of colour development defined as the antioxidative activity (AOA). Different solutions prepared are as shown below:

(1) Sodium phosphate buffer: 100 mmol/litre, pH 7.4
(2) Sodium benzoate: 10 mmol/litre
(3) NaOH: 50 mmol/litre
(4) EDTA (acidum aethylendiamin tetraacetic): 2 mmol/litre in phosphate buffer (solution1)
(5) $Fe(NH_4)_2SO_4$: 2 mmol/litre
(6) Fe–EDTA complex (prepared freshly by mixing equal volumes of solutions 4 and 5, left to stand 60 minutes at room temperature)
(7) H_2O_2: 10 mmol/litre
(8) Acetic acid: 20%
(9) Thiobarbituric acid (TBA): 0.8% (wt/vol) in 50 mmol/litre NaOH
(10) Uric acid: 1 mmol/litre in 5 mmol/litre NaOH.

Solutions 4–9 were freshly prepared before use. The sodium phosphate buffer and sodium benzoate were kept in a refrigerator (4 °C) and the uric acid solution in a deep freeze (−20 to −30°C).

Analytical Procedure

Each sample (A_1) had its own control (A_0, sample blank) in which the Fe–EDTA mixture and H_2O_2 was added after 20% acetic acid. For each series of analysis, a negative control (K_1 and K_0) was prepared (at least in triplicate), containing the same reagents as A_1 or A_0, except that serum was replaced with phosphate buffer. Standards containing 1 mmol/litre uric acid (UA_1 and UA_0) were used for calibration. Pipette into tubes (in millilitres) were as shown below:

	A_1	A_0	K_1	K_0	UA_1	UA_0
Serum	0.01	0.01	-	-	-	-
Uric acid	-	-	-	-	0.01	0.01
Buffer	0.49	0.49	0.5	0.5	0.49	0.49
Na-benzoate	0.5	0.5	0.5	0.5	0.5	0.5
Acetic acid	-	1	-	1	-	1
Fe-EDTA	0.2	0.2	0.2	0.2	0.2	0.2
H_2O_2	0.2	0.2	0.2	0.2	0.2	0.2
Incubated for 60 minutes at 37°C, then the following were added:						
Acetic acid	1	-	1	-	1	-
TBA	1	1	1	1	1	1

The prepared samples were incubated again for 10 minutes at 100 °C (in a boiling water bath) then cooled in ice bath. The total antioxidant capacity of samples was then determined using spectrophotometer with absorbance at 532 nm against deionised water. Antioxidant activity was then calculated as follows:

AOA (mmol/litre) = (CUA) (K − A)/(K − UA)

where

K = absorbance of control ($K_1 - K_0$)

A = absorbance of sample ($A_1 - A_0$)

UA = absorbance of uric acid solution ($UA_1 - UA_0$)
CUA = concentration of uric acid (in mmol/litre).

Insemination procedure

The 75 breeder hens were randomly allotted to the 5 treatments groups and 5 replicates, with 3 birds per replicate in a completely randomized design. The birds were artificially inseminated twice per week (Mondays and Thursdays) by depositing 0.1 mL of extended pooled semen into their oviducts according to their respective treatments. For insemination, while holding the hen upright, the vent was cleaned with the use of tissue paper to remove feacal contamination after which pressure was applied to the abdomen around the vent, particularly on the left side. This caused the cloaca to evert and the oviduct to protrude, so that a syringe or plastic straw was inserted approximately 1 inch (2.5 cm) into the oviduct and the appropriate amount of semen delivered. As the semen was expelled by the inseminator, pressure around the vent was released, which assists the hen in retaining sperm in the vagina or oviduct (Bramwell 2017).

The daily egg collection used for this study commenced a week after first insemination (i.e. after 2 inseminations). Eggs were collected everyday on treatment basis, marked and stored in a cold room for 7 days after which they were set for incubation. Prior to incubation, set eggs were fumigated with formalin and potassium permanganate at ratio 1 to 1 mL/g.

Incubation Temperature was set at 99.6 ^{o}F (37.6 ^{o}C) and relative humidity of 85%. Candling was done on the 18th day inside a dark room to determine fertility using light penetration method. Candling clears were removed. The remaining eggs considered fertile were transferred into the Hatcher and chicks hatched on day 21. Hatcher temperature and relative humidity were 98.5 ^{o}F (36.9 ^{o}C) and 87.5 respectively. Fertility and hatchability expressed in percentage were determined by:

$$\text{Percentage fertility} = \frac{\text{Number of fertile eggs}}{\text{Number of set eggs}} \times \frac{100}{1}$$

$$\text{Hatchability of set eggs} = \frac{\text{Number of hatched chicks}}{\text{Number of set eggs}} \times \frac{100}{1}$$

$$\text{Hatchability of fertile eggs} = \frac{\text{Number of hatched chicks}}{\text{Number of fertile eggs}} \times \frac{100}{1}$$

Statistical Analysis

Data obtained were analysed using one-way analysis of variance procedure of SAS (2003). Means were separated using the Duncan's multiple range test of the same software. Mean values of fertility and hatchability were correlated using Pearson's correlation the same software.

III. Results

Evaluation of the Combined Effects of Selenium and α-Tocopherol on Progressive Motility of Extended (27 to 29 ℃) Cock Semen

The results of the combined 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 difference (p>0.05) among the treatment means across the storage periods up to 6 hours. The highest value obtained at 0 hour is 93.30% in (T2) while the lowest value was obtained at T5 (86.70%). At 3 hours evaluation period, the values obtained ranged from 46.70% in T4 to 56.70% in T2. It was observed that progressive motility decreased with time to 0.00% in T4 and T5 at 6 hours post storage while the highest value was 3.33% at T2. Progressive motility decreased below 50.00% after 3 hours of storage under room temperature except for T4 which was already 46.70% at 3 hours post storage.

Table 2: Combined Effects of α-Tocopherol and Selenium Fortified Extender on Progressive Spermatozoa Motility (%) at Room Temperature

Storage period	Treatments T1 Control	T2 (25% T)	T3 (25% SE)	T4 12.5%(T+SE)	T5 25%(T+SE)	SEM	*p*
0 hour	90.00	93.30	88.30	90.00	86.70	2.24	0.59
3 hours	55.00	56.70	55.00	46.70	50.00	3.42	0.50
6 hours	5.00	3.30	1.70	0.00	0.00	1.29	0.23

Evaluation of the Combined Effects of Selenium and α-Tocopherol on Percentage Liveability of Extended (27 to 29 ℃) Cock Semen

The results of the combined effects of α-tocopherol and selenium fortified extender on percentage liveability of stored semen (27 to 29 °C) are shown in Table 3. There were no differences (p>0.05) among treatment means except at 0 hour. T4 (95.50%) was higher (p<0.05) than T3 (91.20%) and T5 (91.50%) although not different (p>0.05) from T1 (94.00%) and T2 (94.67%). At 3 hours, values obtained range from 81.17% in T5 to 88.00% in T2 while the lowest value obtained at 6 hours was 58.30% in T5 and the highest value was 78.70% in T3. The addition of selenium at 25% inclusion level appears to be toxic at 0 hour. However, reduced combination of 12.5% α-tocopherol and selenium in extender favours percentage liveability of semen stored under room temperature (27 to 29 °C).

Table 3: Combined Effects of α-Tocopherol and Selenium Fortified Extender on Spermatozoa Liveability (%) at Room Temperature

Storage period	Treatments T1 Control	T2 (25% T)	T3 (25% SE)	T4 12.5%(T+SE)	T5 25%(T+SE)	SEM	*P*
0 hour	94.00^{ab}	94.70^{ab}	91.20^{b}	95.50^{a}	91.50^{b}	0.89	0.08
3 hours	84.30	88.00	84.20	87.50	81.20	2.63	0.63
6 hours	76.50	68.20	78.70	71.00	58.30	5.88	0.40

$^{a, b, c}$ Means along the same row without common letter are different at p<0.05

Evaluation of the Combined Effects of Selenium and α-Tocopherol on Total Antioxidant Capacity of Extended (27 to 29 ℃) Cock Semen

The results of the combined 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 differences (p<0.05) among treatment means at 0 and 6 hours storage period. T1 (1.96 mmol/L) and T3 (2.71 mmol/L) were higher (p<0.05) than T2 (1.06 mmol/L) although similar (p>0.05) to T4 (1.86 mmol/L) and T5 (2.40 mmol/L). At 3 hours, the least value was obtained at T4 (1.98 mmol/L) while the highest value was obtained at T3 (2.35 mmol/L). The highest (p<0.05) value obtained at 6 hours evaluation period was at T4 (3.17 mmol/L). Although similar (p>0.05) to T3 (2.07 mmol/L), it differ (p<0.05) from T1 (1.94 mmol/L), T2 (1.73 mmol/L) and T5 (0.85 mmol/L).

Table 4: Combined Effects of α-Tocopherol and Selenium Fortified Extender on Total Antioxidant Capacity (mmol/L) at Room Temperature

Storage Period	Treatments T1 Control	T2 (25% T)	T3 (25% SE)	T4 12.5% (T+SE)	T5 25%(T+SE)	SEM	*p*
0 hour	1.96^{a}	1.06^{b}	2.71^{a}	1.86^{ab}	2.40^{ab}	0.33	0.71
3 hours	2.18	2.25	2.35	1.98	1.99	0.32	0.21
6 hours	1.94^{b}	1.73^{b}	2.07^{ab}	3.17^{a}	0.85^{b}	0.28	0.35

$^{a, b, c}$ Means along the same row without common letter are different at p<0.05

Evaluation of the Combined Effects of Selenium and α-Tocopherol on Lipid Peroxidation of Extended (27 to 29 ℃) Cock Semen

The results of the combined effects of α-tocopherol and selenium fortified extender on lipid peroxidation of stored semen (27 to 29 °C) are shown in Table 5. Lipid peroxidation was not significantly (p>0.05) influenced by the inclusion levels throughout the periods of storage. The values obtained at 0 hour ranged between 0.59 mMMDA/l0^{-6} Spermatozoa (T5) and 1.38 mMMDA/l0^{-6} Spermatozoa (T2). The least value obtained at 3 hours storage period was at T4 (0.39 mMMDA/l0^{-6} Spermatozoa) while the highest value was at 1.28 mMMDA/l0^{-6} Spermatozoa (T5). However, at 6 hours, the least value was at T5 (0.52 mMMDA/l0^{-6} Spermatozoa) while the highest value was at T4 (1.57 mMMDA/l0^{-6} Spermatozoa).

Table 5: Combined Effects of α-Tocopherol and Selenium Fortified Extender on Lipid Peroxidation (mMMDA/l0^{-6} Spermatozoa) at Room Temperature

Storage period	Treatments T1 Control	T2 (25% T)	T3 (25% SE)	T4 12.5% (T+SE)	T5 25%(T+SE)	SEM	*p*
0 hour	0.98	1.38	1.18	1.05	0.59	3.69	0.21
3 hours	0.46	0.59	0.79	0.39	1.28	2.05	0.86
6 hours	1.44	0.52	0.72	1.57	0.85	3.32	0.67

In vivo Evaluation of the Effects of Selenium, α -Tocopherol and their Combined Effect in Extended Cock Semen (27-29 ℃)

Figure 1 presents the effects of α-tocopherol and selenium in extended cock semen (27-29 ^{o}C) on fertility and hatchability of eggs in breeder hen. There were differences (p<0.05) in all the parameters evaluated across the treatments. Fertility rate value was highest in T1 (86.50%) and T3 (86.00%) similar (p>0.05) to T4 (82.00%) but differed (p<0.05) from T5 (72.30%) which was also different (p<0.05) from T2 (57.00%) being the least value. Non fertile eggs were higher (p<0.05) in T2 (43.00%) followed by T5 (27.80%) and T4 (18.00%) and the least (p<0.05) was at T1 (13.50%) and T3 (14.00%). Hatchability of set eggs showed that T1 (69.70%), T3 (71.30%), T4 (69.50%) and T5 (59.00%) were higher (p<0.05) than T2 (40.00%). Hatchability of fertile eggs showed that T1 (80.50%), T3 (82.50%) and T5 (81.50%) although not different (p>0.05) from T4 (79.3%) were higher (p<0.05) than T2 (69.50%). It was observed that fertility, hatchability of set eggs and hatchability of fertile eggs were higher (p>0.05) at T3 (25% inclusion of selenium) and lowest (p<0.05) at T2 (25% inclusion of α-tocopherol) compared to the control.

Table 6 shows the correlation of fertility parameters of extended cock semen fortified with α-tocopherol and selenium and inseminated under room temperature. Positive correlation coefficients were observed for fertile eggs with hatchability of set eggs (95.00%), fertile eggs with hatchability of fertile eggs (65.00%) and hatchability of set eggs with hatchability of fertile eggs (85.00%) while negative correlation coefficients were observed for all parameters correlated with non fertile eggs. Therefore, Lohmann egg hatchability is dependent on fertility which was enhanced by α-tocopherol and selenium in the extended cock semen.

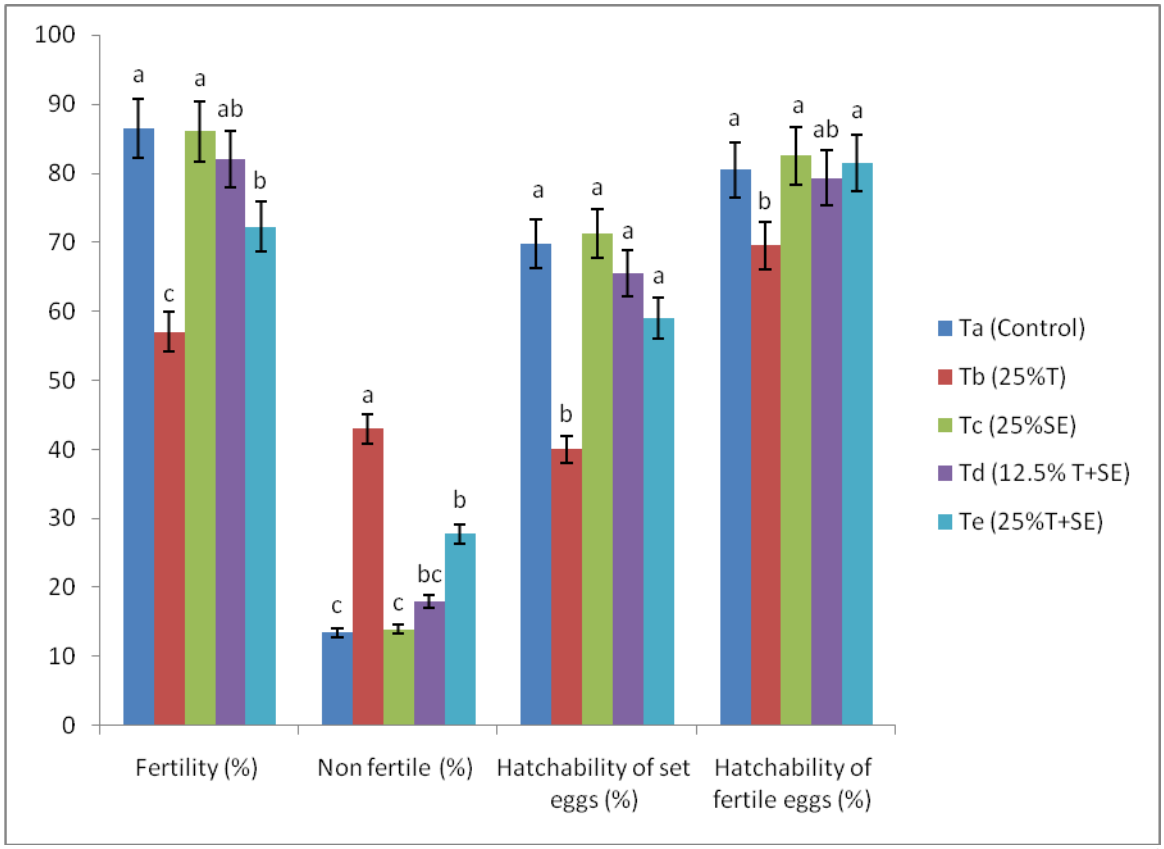

Figure 1*: In vivo* Combined Effects of α-Tocopherol and Selenium in Extended Cock Semen stored at 27-29 ^{o}C on Fertility and Hatchability in Breeder Hen

a, b,c: Bars with different superscripts within parameters are different (p<0.05).
T=Tocopherol; SE=Selenium; T1: Semen + RS (Control); T2: Semen + RS with 25µgmL^{-1} of α-tocopherol; T3: Semen + RS with 25µgmL^{-1} of selenium; T4: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium; T5: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

Table 6: Correlation of Fertility Parameters of Extended Cock Semen Inseminated under Room Temperature

Parameters	Fertile eggs	Non fertile eggs	Hatchability of set eggs	Hatchability of fertile eggs
Fertile eggs (%)	1.00	-1.00*	0.95*	0.65*
Non fertile eggs (%)		1.00	-0.95*	-0.65*
Hatchability of set eggs (%)			1.00	0.85*
Hatchability of fertile eggs (%)				1.00

* There were correlations at p< 0.05

IV. Discussion

Motility was observed to decrease below 50% after 3 hours except at 12.5µgmL^{-1} of α-tocopherol and selenium which decreased below 50% before 3 hours. This was similar to the work of Ajala et al (2012) who reported decline below 60% in motility after 3 hours 30 minutes of incubation of unextended buck semen at room temperature. This could be attributed to the high metabolic activity rate of spermatozoa under the storage condition which may have caused a faster depletion of all available nutrients thus resulting in rapid decline in motility. Also, the presence of exogenous antioxidant probably could not provide the energy required for metabolic activities by the spermatozoa which was deficient in the composition of the conventional extender (Ringer's solution) chosen for this experiment. The energy used by the spermatozoa for the stored period was from the reserve of the Adenosine triphosphate in the mitochondria during the cell formation *in vivo.*

The decline observed for liveability at 0 hour in 25µgmL^{-1} selenium based treatments as compared to 12.5µgmL^{-1} probably suggest that addition of selenium at higher values up to 25µgmL^{-1} could be detrimental to the survivability of spermatozoa cells in relation to lower dosage at the initial hour. This agreed with the work of Dorotskar et al (2012) who reported a deleterious effect of selenium supplementation on spermatozoa parameters as early as 0 hour. However, the high result obtained at 12.5µgmL^{-1} inclusion level of selenium contradicted their result of damages done at 4 and 8µgmL^{-1} at 0 hour. The antioxidant synergy however did not influence the liveability of the spermatozoa.

It was observed from the combined effect of α-tocopherol and selenium on total antioxidant capacity that selenium showed greater potential of being readily available in the seminal fluid immediately it was added into the extender relative to α-tocopherol. The activities of the enzymatic action of selenium dependent GSH-Px reported to be present in fresh semen could be responsible for this immediate release from the binder. Enzymes are known to function as catalyst in biological compositions. This could be the reason why selenium based treatments were observed to decrease as it maintained the balance between the reactive oxygen species with time while the control and α-tocopherol-based treatments initially increased at 3 hours before decreasing at 6 hours.

Despite the variation observed in the total antioxidant capacity, the influence of the exogenous addition of α-tocopherol and selenium were not evident in lipid peroxidation across the treatments throughout the periods of storage. However, the values obtained for control decreased at 3 hours and increased at 6 hours. This could be due to complete depletion of the antioxidants present in the raw semen. On the other hand, addition of α-tocopherol and selenium alone showed gradual decline in values from 0 hour to 6 hours. This could be attributed to the ability of the α-tocopherol and selenium to protect the spermatozoa from peroxidative damages as reported by several authors (Surai et al 2003 and Khan 2011). The inclusion level of α-tocopherol and selenium at 12.5µgmL^{-1} followed the same pattern with the control which suggests inadequate antioxidant content in the seminal fluid of semen samples evaluated over time.

The mechanism of antioxidant defence *in-vitro* varies from that of *in-vivo* (inside the spermatozoa storage tubules (epididymis) of the breeder hens where it will be stored for days). This research work clearly proved that the susceptibility of spermatozoa to peroxidation is a major limiting factor to *in vitro* spermatozoa storage in cock semen despite the addition of α-tocopherol and selenium. The implication of the antioxidants (α-tocopherol, selenium and their synergy) was then studied *in-vivo* using fertility as an indicator.

It was observed that fertility and hatchability of cock semen extended with α-tocopherol inclusion in Ringer's solution was low. This is similar to the report of Long and Kramer (2003) who stated that low fertility of stored turkey spermatozoa was not enhanced by the presence of α-tocopherol. Lipid peroxidation might have adversely affected spermatozoa energetics that in turn affected the fertilising ability of turkey spermatozoa extended and fortified with α-tochopherol. However, fertility and hatchability of cock semen extended with selenium in Ringer's solution was highly competitive with Ringer's solution alone. This confirms the importance of antioxidants such as selenium in improving fertility of extended semen. However, it could also be

that the *in vitro* storage of spermatozoa in the spermatozoa storage tubules of the hen is protected from peroxidation (which results in reduced fertility) through enzymatic defence mechanism activated with selenium than non-enzymatic as in α-tocopherol. This could be justified by the report of Breque et al *(*2003) and Khan (2011) who stated that while α-tocopherol inhibit lipid peroxidation and circumvent the negative effects of peroxidation products in spermatozoa membranes, selenium dependent gluthathione peroxidase (GSH-Px) neutralises the toxic biological substances (by-products) generated by spermatozoa catabolism. Surai et al (1998) demonstrated that inclusion of selenium in male diet increased the concentration of GSH-Px activity in testes, spermatozoa and seminal plasma. It is pertinent to say that Se-GSH-Px represent the total enzymatic activity of chicken spermatozoa which further resulted into increased fertility and hatchability of cock semen extended with Ringer's solution fortified with 25µgmL^{-1} of selenium and inseminated under room temperature. The correlation of fertile eggs to hatchability of set eggs was high (95%) which suggested that semen extension will help retain high productivity of artificially inseminated birds and will be more economically viable to poultry farmers in poultry industry.

V. Conclusion

Cock semen extended with Ringer's solution processed at room temperature (27 to 29 ^{o}C) should not exceed 3 hours before insemination for optimum fertility. Addition of selenium and α-tocopherol to Ringer's solution as semen extender had varied effects on total antioxidant capacity and mitigation of lipid peroxidation during *in vitro* storage. Although their combination did not enhance cock semen *in vitro* at room temperature, selenium inclusion in Ringer's solution at 25µgmL^{-1} under room temperature improved fertility and hatchability in Lohmann breeder hens.

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