EFFECTS OF SELENIUM AND α-TOCOPHEROL ON QUALITY AND FERTILISING ABILITY OF SPERMATOZOA IN EXTENDED COCK SEMEN

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CERTIFICATION

I certify that this study was carried out by Adedamola Abimbola LAWANSON with Matriculation Number 158982 in the Animal Physiology and Bioclimatology Unit, Department of Animal Science, University of Ibadan, under my supervision.

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ABSTRACT

Spermatozoon apoptosis in extended semen is a challenge to artificial insemination in poultry production. Chicken spermatozoa undergo damages *in vitro* due to lipid peroxidation among other factors. Extender supplementation with exogenous antioxidants could mitigate lipid peroxidation and improve semen quality. However, inclusion of antioxidants such as selenium and α-tocopherol in semen extender to improve quality of sperm cells has not been adequately documented. 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 30 weeks, pooled and divided into nine groups. Each group was extended with Ringers Solution (RS) without antioxidant (T1), RS with α-tocopherol at 25 (T2), 50 (T3), 75 (T4) or 100µgmL^{-1} (T5) and selenium at 25 (T6), 50 (T7), 75 (T8) or 100µgmL^{-1} (T9). Another set of semen was harvested, pooled and extended with RS (T_A), RS+25µgmL^{-1} α-tocopherol (T_B), RS+25µgmL^{-1} selenium (T_C), RS+12.5µgmL^{-1} α-tocopherol+12.5µgmL^{-1} selenium (T_D), RS+25µgmL^{-1} selenium+25µgmL^{-1} α-tocopherol (T_E) were assessed *in vitro* and *in vivo*. All treatments were evaluated at room temperature (RT: 27-29 ^{o}C) and refrigeration (4-6 ^{o}C) at interval of 3h until motility dropped below 50%. 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 RT and another seventy-five under refrigeration 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 $\alpha_{0.05}$.

At RT, the highest SPM (91.7±1.1%) and SL (94.5±1.0%) at 3h were recorded in T5 and least at T7 (78.3±1.1%) and T9 (83.2±1.0%), respectively. The TAC ranged from 2.0±0.14 (T9) to 3.2±0.14 (T5), while LP ranged from 0.5±0.28 (T1) to 2.8±0.28 (T7). Under refrigeration, SPM was lower in T4 (55.0±0.8%) than other treatments at 6h. The

highest SL (95.7±1.5%) was recorded in T3 and least in T6 (80.8±1.5%), while TAC was highest in T9 (2.7±0.07) and least in T1 (1.9±0.07). Effect of α-tocopherol and selenium combinations under RT was similar among treatments for SPM, SL and LP up to 6h. Under refrigeration, SPM and SL were highest at T_E (95.0±1.9% and 96.5±1.1%, respectively) and least at T_D (76.7±1.9% and 87.0±1.2%, respectively), while TAC and LP were unaffected by the combinations. At RT, egg fertility was higher at T_A (86.5±7.3%), T_C (86.0±7.6%) and T_D (82.0±5.0%) than T_B (57.0±7.1%) and T_E (72.3±8.7%), while hatchability of set eggs ranged between 40.0±8.8% (T_B) and 71.3±10.2% (T_C). Fertility positively correlated with hatchability (r=0.95). Under refrigeration, egg fertility ranged between 35.3±12.4% (T_B) and 54.3±10.0% (T_D), while hatchability was lowest at T_A (20.3±6.8%) and highest at T_D (49.0±14.8%).

α-tocopherol at 100µgmL^{-1} enhanced cock semen quality, but combination of α-tocopherol and selenium did not *in vitro* at room temperature. Selenium inclusion at 25µgmL^{-1} under room temperature improved hatchability in Lohmann breeder hens.

Keywords: Artificial insemination, Lipid peroxidation, Selenium, α-tocopherol, Egg fertility.

Word count: 500

DEDICATION

I dedicate this thesis to Almighty God for the gift of life and the grace to achieve; to my lovely wife for her maximum support and to my parents for their unrelenting and sacrificial scholarship from nursery to Ph.D. level.

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Rapid human population growth and low protein intake are part of the major problems facing developing countries like Nigeria. With a population of about 174 million (Population Reference Bureau, 2013) and with over 70% of the population living below a dollar per day (Watts, 2006), Nigerians have a variety of farm animals with poultry being the highest in number (Adeleke *et al*., 2010). Poultry offers an avenue for speedy transformation in animal protein consumption. The average Nigerian consumes about 9 g of animal protein per capita per day as compared to over 50 g per capita per day in North America and Europe (Boland *et al*., 2013). In the year 2013, the poultry industry in Nigeria is estimated to worth ₦80 billion ($600 million) and it comprises approximately 165 million birds (FAO, 2013).

It has been well established that artificial insemination (AI) in avian species has relative advantages when compared to natural mating (Penfold *et al.,* 2000). These benefits include increased quantity of settable eggs, better overall fertility and hatchability, thereby reducing the cost of production of each day-old chick (Brillard, 2003). Several factors must be synchronized for optimum success of artificial insemination and they include breeder stock management, spermatozoa quality and quantity, spermatozoa dosage, depth of insemination, rate of recurrence and time of artificial insemination (King *et al*., 2002). The increasing use of artificial insemination in the poultry industry necessitates the need for the distribution of good quality spermatozoa (Dumpala *et al.,* 2006). In order for the poultry industry to take advantage of modern artificial insemination procedure, there is necessity to store poultry semen properly. The extension of semen with proper diluents is required prior to AI and storage because chicken semen

is highly concentrated and is of low volume. Several factors help to maintain the quality of semen during storage. Among such factors are diluents used for semen extension, storage conditions such as time, aeration, and holding temperature (Dumpala *et al.*, 2006).

Anghel *et al*. (2009) reported that since the development of large scale AI programs in the twentieth century, the need to inseminate large numbers of females with semen from genetically superior males required transport of semen from the collection point or centre to the site of the female and spermatozoa preservation. The aim of storing spermatozoa is to prolong their fertilising capacity. The preservation of semen for short-term (liquid) storage was achieved by reducing the metabolism of spermatozoa through reduction in storage temperature, and for long-term (frozen) storage by arresting the metabolism at sub-zero temperature. Although the fertilising capacity of spermatozoa may be prolonged by storage in a liquid or frozen state, the storage processes certainly reduce the percentage of motile spermatozoa resulting in degenerative changes to spermatozoa membrane integrity, which eventually reduces fertilising capacity after insemination. Dumpala *et al.* (2006) reported that one phenomenon that appears to be consistent in results with liquid semen storage is the decline in fertility with semen stored longer than 6 hours after the first 5 to 8 weeks of egg production. Over the course of egg production, the efficiency of the spermatozoa storage tubules (SST) reduces, therefore, late season declines in fertility and hatchability are not unusual even with fresh inseminations.

Regardless of the nature of the extenders, motility and membrane integrity of spermatozoa are deteriorating during the cooling process and storage at low temperatures. The degenerative changes are possible results of lipids peroxidation or excessive production of reactive species of oxygen (ROS) (Anghel *et al*., 2009). Lipid composition of semen is exclusive in its content of long chain polyunsaturated fatty acids (LCPUFAs) and these are important components of all cell membranes which cause many bioactive molecules (e.g. eicosanoids). As a result of the highly unsaturated lipids, spermatozoa are very vulnerable to peroxidative damage caused by the actions of free radicals and reactive oxygen species (Zaniboni *et al*., 2006). Oxidative stress is identified to play a

role in the etiology of defective spermatozoa function through mechanism which involves the initiation of peroxidative damage to the plasma membrane. This often leads to a subsequent reduction of spermatozoa progressive motility and a decline in quality resulting in inadequate viable spermatozoa and subsequent reduction in fertility (Purdy *et al.*, 2004; Boonsorn *et al.*, 2010). The use of cold-stored semen is limited by its comparatively short time fertilising capacity. The oxidative damage of spermatozoa during storage is a probable cause of the reduction in motility and fertility during chilled storage of semen (Tabatabaei *et al*., 2011).

Natural antioxidant and synthetic phenolic antioxidant have been reported to be a protective means of preventing lipid peroxidation (LP) of spermatozoa (Shoae and Zamiri, 2008). Thus, supplementation with natural antioxidants or synthetic antioxidants could reduce the impact of oxidative stress during the spermatozoa storage process, thereby improving the quality of chilled semen. Several Antioxidants have been used in order to obstruct or avoid oxidative stress in a lot of cellular organism. These antioxidants act in various ways to either scavenge reactive oxygen species or avoid the negative impact of lipid peroxidation on cell integrity (Baran *et al*., 2009). According to Bucak *et al*. (2007), some antioxidants for example tocopherol, glutathione and vitamin C in association with other semen antioxidant enzymes form antioxidant system which made it competent for shielding spermatozoa from free radicals and toxic substances produced from their metabolic activities.

As reported by Traber and Atkinson, (2007), 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. Also, supplementation with exogenous antioxidant such as tocopherol may possibly lessen the damages done by oxidative stress for the period of spermatozoa storage and retain the quality of extended semen (Michael *et al*., 2009). Selenium is also an essential component of glutathione peroxidase (GSH-PX). Glutathione peroxidase is an enzyme that shields cellular components from free radicals and also serves as an antioxidant for cell membrane lipids (Sanchez-Gutierrez *et al*., 2008). In cellular culture, Zhang *et al.*

(2006) stated that selenium in the form of selenite helps to detoxify the cellular components so as to protect them from oxidative damages.

1.2 Justification of the study

Despite the advances in the poultry industry, the commercial use of extended poultry semen is still unavailable or very limited in Nigeria. This could be due to the fact that storage of extended poultry semen is not guaranteed, or available resources have not been exploited for the purpose. The susceptibility of chicken semen to lipid peroxidation caused by activities of free radicals during storage requires the inclusion of antioxidants in extending media to maintain the quality of spermatozoa for improved chick production. Also, there is dearth of technical information on the use of tocopherol and selenium as antioxidants to fortify conventional extenders for storage of poultry semen. Availability and affordability of the tocopherol and selenium qualify them for consideration for research and possible recommendation for poultry semen preservation. Therefore, assessment of the effect of antioxidants such as tocopherol and selenium at various inclusion levels on extended chicken semen and its influence on egg fertility is pivotal to increased utilization and commercialisation in the poultry industry.

1.3 Objectives of the study

The general objective of this study was to investigate the effect of selenium and α-tocopherol in extended cock semen on quality and fertilising ability of spermatozoa.

Specifically, this study attempted to:

1. Evaluate spermatozoa quality and determine the optimum inclusion concentration of selenium and α-tocopherol in cock semen extended with conventional diluents;

2. Assess the synergistic effect of selenium and α-tocopherol as antioxidants on spermatozoa quality in extended cock semen and,

3. Determine the fertilising ability of spermatozoa in cock semen extended with a conventional extender supplemented with selenium and α-tocopherol.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Poultry production

Agricultural development is generally believed to be a panacea for sustainable development of any nation (Barbu and Capusneanu 2012). The oil boom which would have been an enduring blessing to Nigeria has regrettably necessitated great shift of attention to oil money, which resulted to a total neglect of agriculture. Most empirical evidences suggested that agriculture-led growth offers an unusual opportunity for broad-based poverty reduction (Diao *et al*., 2012). The recent focus on agriculture by the Nigerian government as a cultural sector has the capacity to boost the economy and also create a wide range of employment opportunities. This shows an indication of the recognition of agro – business as a reliable platform for sustainable development (El-Rufai, 2011). According to Adesina (2013), shift from the agricultural sector, and the dependence of Nigeria on solely crude oil, has been detrimental to the development of Nigeria as a nation. The Nigerian government in an attempt to save the economy from obvious economic instability is becoming directly involved in commercial agriculture (Olokundun *et al.,* 2014).

The poultry industry has the most viable market of all the agricultural industry in Nigerian. 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). Advancement of the poultry industry has been portrayed as the fastest means of supplying the protein deficiency prevalent in most developing countries. If the poultry industry is properly

harnessed, it can provide a means of foreign earnings to complement crude oil which presently makes up the major supply of foreign earnings in Nigeria (Poultry site news 2009).

The word “poultry” is relevant to chicken or domestic fowl among others. The domestic fowl is the most common avian species reared in many countries such as Nigeria for either table meat, eggs or both. Poultry industry has grown into a business venture which involves birds in their thousands (Nwandu *et al.*, 2016). Large commercial poultry units has been used to substitute the backyard poultry units with more efficient breeds of egg or meat type birds, improved poultry equipment, balanced feed and intensive housing system. Nonetheless, commercial poultry farming has not been fully developed in the tropics as compared to the temperate regions (Oyeyinka *et al.*, 2011).

Challenges of global food insecurity and hunger most especially in developing countries like Nigeria have receive continuous attention from professionals and governments (Emaikwu *et al.*, 2011). Consequently, quite a lot of conferences and World Food Summit on human nutrition have brought to the fore; discussions on issue of eradicating poverty and hunger. FAO (2013) affirmed that, the most critical in the global food basket crisis is animal protein. Poultry production being a major aspect of livestock production is imperative to the biological needs, economic and social development of the populace in any country (Oladeebo and Ambe-Lamidi, 2007). Nevertheless, the input of poultry production (meat and eggs) to entire livestock output improved from 26% in 1995 to 27% in 1999 with an increase in egg production alone accounting for about 13% throughout the period (Ojo, 2003). In year 2013, the Nigerian poultry industry was anticipated to worth ₦80 billion ($600 million) which consist of about 165 million birds which produces 290,000 metric tons of poultry meat and 650,000 metric tons of eggs. Taking a viewpoint from the market size, Egg production in Nigeria is the leading in Africa (after which South Africa is next with 540,000 metric tons of eggs) and it has the next biggest chicken population following South Africa’s 200 million birds. (Sahel, 2015).

Poultry production is a preferred animal source food because livestock production has been reported to be increasingly affected by carbon emission constraints leading to

environmental and animal welfare legislations (Paint, 2011; Mengesha 2012). However, poultry production has a relative advantage over others due to its little contribution to global warming (FAO, 2010) whereas; ruminants were reported to cause green house gas emissions (Haagsman *et al*., 2009). Furthermore, ILRI, (2006) reported that the genetic diversity of local chicken was much higher than other livestock species because of its good adaptability to climate change and diseases outbreak. Resultantly, the desire for poultry meat and eggs without any taboo and the relative ease in establishing poultry industry is the driving force at the movement (FAO, 2011c). According to FAO (2009) report, chicken was reported to be the cheapest of all domesticated livestock products.

2.2 Poultry reproduction

The most essential index of poultry breeding is poultry reproduction. In female birds, sexual maturity attainment can easily be identified superficially as age at which hens lay their first egg whereas, characterisation in male is internal (Tadondjou, 2014). In male birds, age at sexual maturity, fertility and spermatozoa storage time in female genital tract are indices by which reproduction is characterised (Addisu, 2013).

Fertility is one of the most major economic traits in poultry production, together with egg hatchability. Male fertility potential may be defined as the ability to produce and ejaculate spermatozoa that are capable of fertilising eggs. This include completion of all the steps in the fertilisation process i.e. spermatozoa movement across the female reproductive tract and getting to the Spermatozoa Storage Tubule (SST), penetration into the perivitelline layer, syngamy and fertilisation (Celeghini *et al*., 2001). In all avian species, spermatozoa qualities decrease with age leading to an increasing decline in fertility rate. Nonetheless, the rate of decline is dependent on strain and species (Ilori *et al.,* 2012). According to Bah *et al.* (2001), it was reported that fertility rate of some Nigerian local chicken was as low as 45.8%, while FAO (2004) reported fertility rate ranging from 53 to 60% and 55 to 90% in local chicken in Ethiopia and Southern Asia respectively. The length of fertility is extremely important because spermatozoa are stored in the utero-vaginal glands of female birds for a long time (Blesbois *et al.*, 2007). Avians have a specialized section at the upper 2cm of the vagina, closest to the uterus

called the utero-vaginal junction. As reported by Miller (2011), the internal layer of the utero-vaginal junction is made up of spermatozoa storage tubules which helps to preserve spermatozoa for several days to some weeks, during which they are gradually released to swim up the oviduct and fertilise ovulated ova. Spermatozoa storage is dependent upon a number of factors among which is spermatozoa motility which helps retain their location contrary to the outflow of fluid from the spermatozoa storage tubules (Froman *et al.*, 2011). The location and parts of the reproductive organs in the male and female chicken is presented in figures 1 and 2 respectively.

In the hen, it is the left oviduct and ovary that are functional. Approximately 2 to 3 weeks prior to the beginning of lay, white-yolk follicles (less than 1 mm in diameter) start to accrue yolk in which they are being released in order of hierarchy of growing yolk follicles. When it is time for ovulation, the most mature follicle often chosen as F1 is ovulated. The yolk follicle matures at about 17 days and grows to preovulatory 40 mm diameter yellow yolk follicle (Bakst and Dymond, 2013). Immediately the follicle designated as F1 is ovulated, the next most matured follicle, chosen as F2 will be converted into the F1 follicle and ovulate at the onset of the next ovulatory cycle (24 to 26 hours).

The follicular covering that surrounds the growing oocyte is made up of separate concentric layers of cells namely the outer serosa also known as the germinal epithelium and the theca externa, that becomes the largest segment of the follicular epithelium which supplies structural hold to the follicle and has steriodogenic cells. Others are the theca interna, a highly vascularised layer, similar to the theca externa which are made up of cells that produce steroid (both thecal layers produce estrogens, androgens and the granulosa cell layer covering the oocyte, which is in charge of secreting progesterone and the production of the internal perivilline layer). The internal perivilline sheath is similar to the zona pellucida in mammals and is a tough reticulum of approximately 2 μm in thickness. During ovulation, only the internal perivilline layer surrounds the ovum. Because corpus luteum is not formed in avian species, the granulosa and the thecal layers of the post-ovulatory follicle generate progesterone and prostaglandins respectively, and

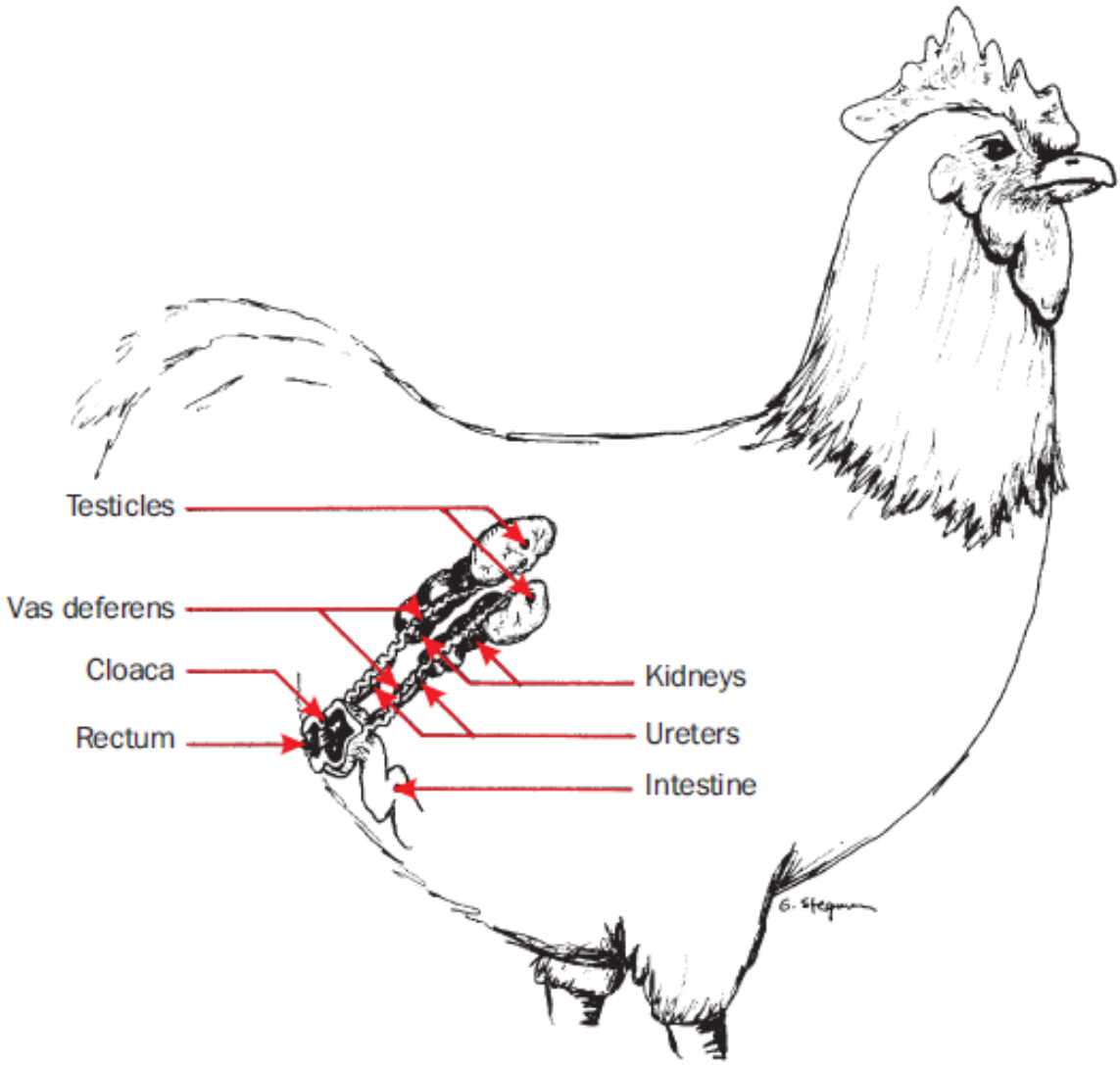

Figure 2.1: Cock showing the location and parts of reproductive system

Source: Reproduction in Poultry (2016)

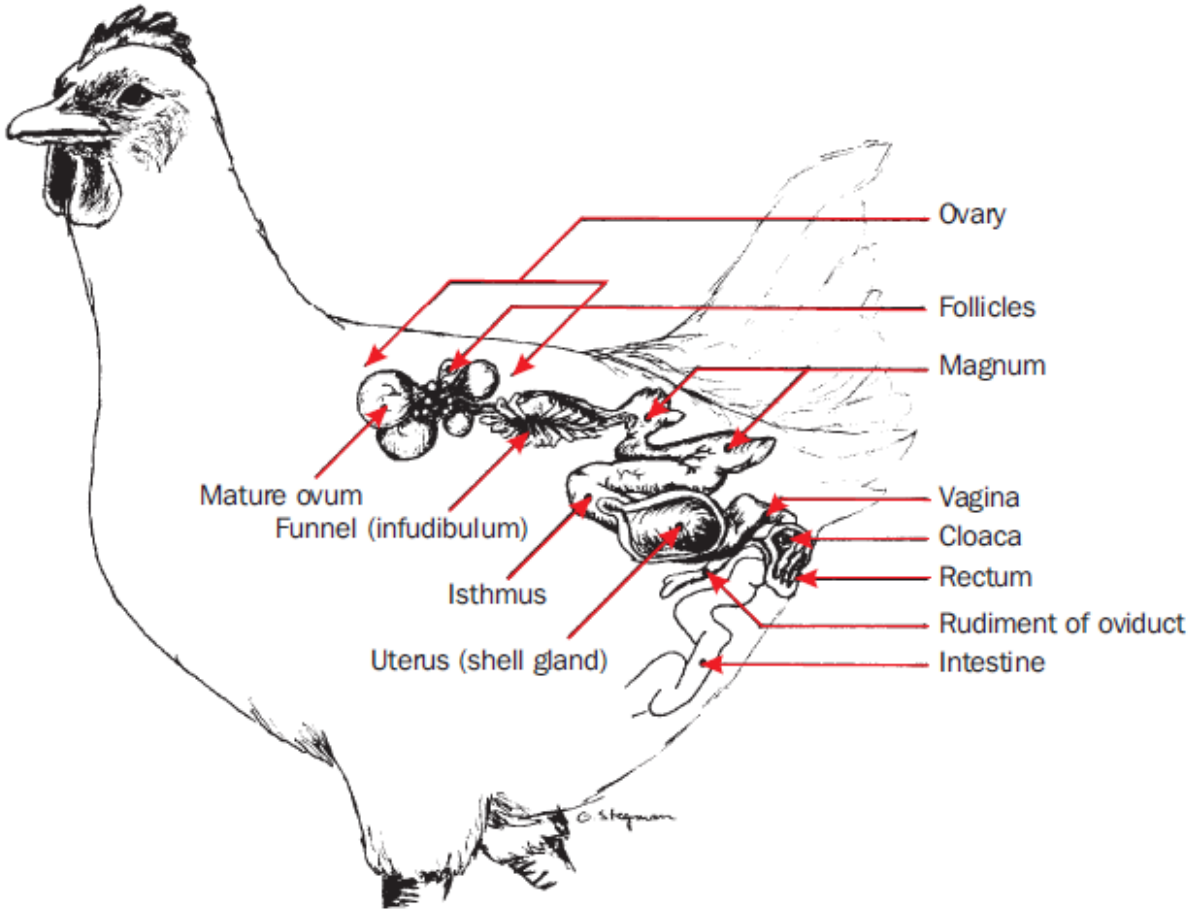

Figure 2.2: Location and parts of reproductive system in a hen

Source: Reproduction in Poultry (2016)

then degenerate within 72 hours (Johnson and Woods, 2007). After ovulation, the appearance of the post-ovulatory follicle is like pockets. Superficially, the internal perivilline layer covering the germinal disc, with a 3.5 mm thick disc of white yolk having the haploid pro-nucleus and related organelles, are spermatozoa receptors. Spermatozoa attach to the receptors covering the germinal disc, hydrolyse a pathway via the internal perivilline layer, after which they penetrate into the ovum. Polyspermy is natural in avian although only a single spermatozoa in apposition to the female pro-nucleus goes through nuclear de-condensation and initiates syngamy, thereby forming the diploid number of chromosomes (Bakst and Dymond, 2013).

2.3 Cock Semen

2.3.1 Characteristics and chemical components of cock semen

Cock semen is made up of spermatozoa and seminal plasma both of which are produced in the testes as peculiar to avian species. The endocrine hormones transported through the blood stream helps to regulate these secretions in the testes. Follicle stimulating and luteinizing hormone secreted from the pituitary gland controls the testes, which sequentially produces testosterone that regulates testicular secretions and development (Hafez, 1974).

Table1 shows the characteristics and average chemical composition of semen in a cock as reported by Getachew (2016).

Table 2.1: Characteristics and mean chemical composition of cock's semen

Semen	Quantity
Calcium (mg/100mL)	10
Chloride (mg/100mL)	147
Ejaculate volume (mL)	0.2 - 0.5
Ergothioneine (mg/100mL)	0 - 2
Fructose (mg/100mL)	4
Glycerylphosphoryl choline (mg/100mL)	0 - 40
Inositol (mg/100mL)	16 - 20
Magnesium (mg/100mL)	14
Morphologically normal spermatozoa (%)	85 - 90
Motile spermatozoa (%)	60 - 80
pH	7.2 - 7.6
Potassium (mg/100mL)	61
Protein (g/100 mL)	2.8
Sodium (mg/100mL)	352
Sorbitol (mg/100mL)	0 – 10
Spermatozoa concentration ($\times 10^6$ /mL)	3000 - 7000
Spermatozoa/ejaculate ($\times 10^9$)	0.06 - 3.5

Source: Getachew (2016)

2.3.2 Semen Evaluation

Semen evaluation techniques are methods used for assessing the quality and quantity of semen parameters. According to Madhuri *et al.* (2012), for a laboratory analysis to be useful in semen evaluation, it must be repeatable, objective, accurate, rapid and inexpensive to evaluate.

Macroscopic test are evaluations done without the aid of a microscope and results can be seen with ordinary eyes. They are often referred to as traditional semen evaluation techniques. Among the parameters that can be assessed using macroscopic test are semen volume, semen colour, consistency of the semen, semen density and semen pH. Semen volume, colour and consistency are observed visually with analytical value to a certain extent indicating the functionality of the animals' accessory glands such as the possible spermatozoa concentration (Madhuri *et al.*, 2012).

Ejaculate volume: The cock releases between 0.1 ml and 1.5 mL during each ejaculate with about 0.6 mL being the mean ejaculate volume as reported by Cole and Cupps (1977). Different volumes of semen are produced by different cocks of the same species at different times (Anderson, 2001). Even a single cock does not produce the same quantity of semen when ejaculated. Gordon (2005) reported an average semen volume of approximately 0.25mL using the abdominal massage technique while Bah *et al*. (2001) reported an average semen volume of 0.28 ± 0.14mL for cock. Nevertheless, Peters *et al*. (2008) recorded cock semen volume that ranged between 0.37 ± 0.02 to 0.73 ± 0.01 ml. According to Senger (2003), it is imperative to note that spermatozoa concentration and semen volume will determine the total number of spermatozoa collected per ejaculation which are important criteria for determining the quantity of insemination doses that can be gotten from each ejaculate.

Semen colour: The semen of the chicken ranges between an intense thick fluid and a diluted liquid produced by different reproductive glands. It ranges between a relative high spermatozoa concentration of clear and milky white containning reduced number of spermatozoa (Peters *et al*., 2008). Colour of semen may possibly also serve as an

indicator of contamination by urine, faeces or blood and making it to be reddish, brownish or greenish (Lake, 1983). Occasionally, there may be traces of blood in the semen collected which may be a result of infection in the reproductive tract, injury or excessive force during the collection process (Bearden *et al*., 2004).

Semen density: The colour of semen is usually used to evaluate the density of an ejaculate. The density can be calculated by multiplying the total spermatozoa concentration (defined as the total number of spermatozoa in the entire ejaculate) by the semen volume (World Health Organisation, 2010).

Semen pH: The value of semen pH indicates its quality. Good quality semen is always neutral or slightly alkaline while poor quality semen is generally acidic in nature. The pH of semen varies to some extent among animal species and diverse breeds. The best semen pH which favours spermatozoa varies between 7.0 and 7.4. According to Latif *et al.* (2005), spermatozoa motility is usually high within a pH of 7.0 and 7.4 resulting in increased fertility, compared to a pH of 6.4, which may lead to alteration of the membrane integrity of the spermatozoa during semen preservation. A contrasting report by Donoghue and Wishart (2000) showed from several trials indicating that cock spermatozoa can tolerate a pH range of 6.0 to 8.0 while Bah *et al, (*2001) reported a semen pH as high as 7.54 ± 0.04 to 7.80 ± 0.03.

Progressive motility: Microscopic spermatozoa motility estimation is an entirely subjective evaluation and is determined by the experience and ability of the person, however, it is the single most used parameter to determine the quality of semen planned for artificial insemination (Madhuri *et al.* 2012). The viability and fertilising ability of spermatozoa in ejaculated semen are mainly judged by progressive motility (Etches, 1996). Evaluation of spermatozoa motility is evaluated with fresh and diluted semen (Hafez and Hafez, 2000). Assessment of undiluted semen indicates the quality of the spermatozoa in its seminal fluid, which often makes individual spermatozoa motility patterns difficult to distinguish when spermatozoa concentrations are high, hence, the

need for extension of semen aliquot prior to motility evaluation (Hafez and Hafez, 2000). Individual motility checks the progressive movement of sperm cells.

Semen concentration: Hafez and Hafez (2000) stated that semen collected from domestic cock contains an average spermatozoa concentration of 3 - 7 × 10^9 sperm/mL. On the other hand, the report of Gordon (2005) stated that semen collected from domestic cock contains an average spermatozoa concentration of 5 × 10^9 sperm/ml. The initial method used in most centres is to estimate the spermatozoa concentration with the use of haemocytometer or specialised spectrophotometer which works by determining the turbidity of a measured diluted semen sample (Donoghue *et al*., 1996). Auger *et al*. (2000) have demonstrated vividly that the results are difficult to compare because of variations between laboratories and between technicians. All these had led to the increased use of flow cytometry to estimate spermatozoa concentrations and to bring concurrent agreement between different laboratories (Eustache *et al*., 2001). Other recent facilities developed for determining spermatozoa concentration are microcell analysis, fluorescent plate reader, image analysis and flow cytometer (Tsuji *et al.*, 2002)

Liveability is done to determine the population of spermatozoa in an ejaculate that is still alive to fertilise an ovum. It is done by mixing a drop of semen sample with a drop of the eosin nigrosin stains on a pre-warmed slide using applicator stick and a thin smear is made using another slide (Khan and Ijaz 2008). After air-drying, the smear is observed under a phase contrast microscope (X40 magnification) for unstained heads of the spermatozoa (live) and stained or partial stained heads of the spermatozoa (dead). A total of 200 spermatozoa are counted to determine the percentage of live and dead spermatozoa. The mean of 3 observations must be considered as a single data point.

Morphology: Reproductive ability in the male comprises the production of semen containing normal spermatozoa (quality) in the adequate number (quantity), together with the desire and ability to mate (Oyeyemi and Ubiogoro, 2005). Normally, a spermatozoon has a head, midpiece and tail region. The head region encloses the nucleus containing the genetic material, which is the sire's genetic donation to the offspring (Tuncer *et al*.,

2006). Morphology is usually examined with an eosin-nigrosin stain (background stain) to highlight the cells. The final slide should have dark and light areas that allow you to view different coloured backgrounds as needed when examining the slide. Examine the cells under X1000 magnification (oil immersion) to fully assess the morphology (Madhuri *et al*., 2012). The acrosome (which covers the anterior part of the nucleus) and post-nuclear cap (covering the posterior part of the nucleus) both protects the nucleus. when the acrosome is damaged or malformed, the spermatozoa cell will not be capable of penetrating the zona pellucida and fertilise the ova. Tuncer *et al*., (2008) recorded values of acrosome, spermatozoon head, middle piece and tail deformations of 0.39 ± 0.03%, 1.06 ± 0.03%, 2.32 ± 0.05% and 2.53 ± 0.04% respectively in cocks.

Sperm morphology is one of the parameters used to estimate semen quality and limitations in the male. The success of the evaluation method is dependent on the preparation of the stain used. In advanced laboratories, the use of computer assessment semen analyser is being adopted for spermatozoa assessment. Eosin-nigrosin is a stain generally used to determine abnormalities by smearing it on a glass slide immersed in oil and viewed under the light microscope. According to Lukaszewicz *et al*. (2008), Live, damaged, properly formed, viable and non-viable spermatozoa can be estimated using this evaluation technique. The morphology of spermatozoa in avian semen varies from that of mammals. Nevertheless, variations also occur among poultry species, although the size and shape of the spermatozoa are alike. In avian, the spermatozoon is bounded by the cytoplasmic membrane and the acrosome has an internal spine covered by a conical shaped head. Hafez (1974) highlighted that the spermatozoon cap encloses the nuclear substance of the gamete, whereas the mid-piece is made up of the cylindrical centrioles encircled by a sheath of mitochondria. The mid-piece of cock spermatozoon is significantly elongated in comparison with other species, about a quarter longer and this attribute makes cock spermatozoon to exhibit more mid piece bending than other species. According to Alkan *et al*. (2001) the *in vitro* assessment morphological spermatozoa defects of cock semen include: total head swelling, acrosome damage (swelling, rounding, bending or knotting), mid piece damage (mid-piece bending), and tail defects.

An accurate morphological examination of spermatozoa thus enables the elimination of males with potentially low fertility prior to the preservation of their semen (Rodriguez-Martinez and Barth, 2007).

Plasma membrane integrity: The spermatozoon membrane is directly or indirectly related with many spermatozoal functions, warranting the capability of the cell to maintain homeostasis and depict motility and the capacity to interact with the environment, including the lining epithelium of the female genital tract or the oocyte-cumulus cell-complex (Rodríguez-Martínez, 2003). Even though the spermatozoon plasma membrane envelops the whole cell, it comprises of various distinctive membrane sections, such as the outer cover of acrosome membrane, those that cover the post acrosome portion of the spermatozoa head down to the annulus, the principal piece and the rest of the tail (Hossain *et al*, 2011). The integrity of spermatozoa plasma membrane being intact is of utmost importance for cell function (Rodríguez-Martínez, 2003). The ability of plasma membrane to be stable and permeable are two directly related functions, hence assessment of membrane permeability explains its association (Reber *et al.*, 2002), and its competence to go through biophysical alteration such as fluid content and the penetration of cryoprotectant agents during cryopreservation (Peña, 2007). Cryopreservation sometimes causes permanent destruction to the plasma membrane resulting into cell mortality of so many spermatozoa (Holt, 2000). Consequently, it is essential to know the permeability coefficient of the cells to cryoprotectant agents, in addition to their consequences on the hydraulic conductivity of the membrane. Various methodologies such as electron microscopy, coulter counter, differential scanning calorimetry and stopped-flow fluorometry have been used for years to assess membrane permeability (Agca *et al.*, 2005).

Acrosome integrity: The acrosome is a membrane-enclosed structure covering the anterior part of the spermatozoon nucleus. Powerful hydrolysing enzymes are synthesized in that structure, which is a basic feature of the spermatozoa head of all animals (Yanagimachi, 1994). Acrosomal integrity is a requirement for fertilisation (Rodríguez-Martínez, 2006), necessary for spermatozoon infiltration into the zona pellucida.

Biologically, the plasma membrane and the superficial acrosome membrane fuse and vesicate during the acrosome reaction when activated by spermatozoa binding to the zona pellucida. Acrosome integrity is conventionally observed *in vitro* with the use of differential interference-contrast microscopy on unstained samples or phase-contrast microscopy or light microscopy for stained samples (Rodríguez-Martínez, 1997)

2.4 Fertility and Hatchability of Breeder Eggs

Success of poultry industry irrespective of the scale of production is dependent on continuous supply old day-old chicks. The production of day-old chicks is affected by the fertility and hatchability of the eggs which are further influenced by both environmental and genetic factors (King'ori, 2001). Some of those factors include nutrition, breed, age and health status of birds, stress level of the birds, photo-periodism, temperature-humidity index for birds and during incubation e.t.c. Increased production of day-old chicks starts with proper selection and management of breeder stock to proper post-lay handling of fertile eggs in between which there could be semen processing and artificial insemination. Also, successful fertilization in birds occurs following a culmination of a series of events between properly grown breeder males and females. These events, in order, are: the physical act of mating (or collection of semen, processing and insemination in the case of artificial insemination), sperm storage within the hen, sperm transport within the oviduct, recognition of and penetration through the wall of the ovum, and the successful joining of the male and female gamete (Bramwell 2002).

2.5 Artificial Insemination

Artificial Insemination (AI) is the manual placement of semen into the reproductive tract of the female by a technique other than natural mating. It is one of the various advanced technology generally referred to as Assisted Reproduction Technologies (ARTs), through which offspring are produced by aiding the union of gametes (spermatozoa and ova). Assisted reproductive technologies may also entail the relocation of the products of gamete fusion to a female, for example, if fertilisation has taken place *in vitro* or in another female. Other techniques encompassed by Assisted Reproduction Technologies include: intra cytoplasmic spermatozoa injection (where a single spermatozoon is caught

and injected into an ovum); *in vitro* fertilization (where fertilization takes place outside the body); gamete intra fallopian transfer (where spermatozoa are injected into the oviduct to be close to the site of fertilization *in vivo*); embryo transfer (where embryos that have been derived either *in vivo* or *in vitro* are transferred to a recipient female to establish a pregnancy) and cryopreservation (where spermatozoa, ova or embryos are stored in liquid nitrogen) (Morrell, 2011).

Artificial insemination is considered the first reproductive biotechnology, created with the major intention of controlling the dissemination of venereal diseases. AI is still the main vehicle for rapid dispersal of desirable genes and has been the method of choice for the farmers around the world to improve the genetic potentiality of their livestock (Vishwanath, 2003). Artificial insemination has been in use with various familial species which include bees and human beings. It is the most frequently used among artificial reproductive technologies in farm animals, modernising the animal breeding industry during the 20th century contrary to medical use, where intra-uterine insemination is used only occasionally in human fertility treatment. Artificial insemination is undoubtedly the most frequent technique of domestic animals breeding as typified in reproduction of dairy cattle (about 80% in North America and Europe), turkeys (approximately 100% in intensive rearing) and swine production (over 90% in North America and Europe). Artificial insemination 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).

Management of the animal for reproductive soundness is one of the most important factors in goat breeding. Artificial insemination plays an important role in breeding especially in intensive system of production where it can be used as a tool to control reproduction and to improve production (Baillargeon, 2000). The success of artificial insemination programme depends on the appropriate management of semen collection, storage and use (Etches, 1996). Artificial insemination holds several genetic and

economic advantages (Bale, 2003). It also extends the usefulness of proven sires that for some physical reasons or age are unable to copulate normally (Bale, 2003).

2.5.1 Artificial Insemination in Poultry

Assisted Reproduction Technologies (ART's) such as artificial insemination helps to improve poultry production, because it permits a broader use of cocks that are genetically superior with higher productive traits. The development of AI technique has allowed rapid dissemination of genetic materials from a small number of superior sires to a large number of females (Vishwanath and Shannon, 1997). AI is an integral part of poultry reproduction in intensive poultry management. For genetic improvement, AI is regarded as an important breeding tool throughout the world (Tarif *et al,* 2013). Poultry farmers in the years past have 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, *et al.,* 2013). Basically, artificial insemination involves two steps: collection of semen from the male animal and depositing the semen into the female's reproductive tract. Generally, based on the intention and target of the farmer or laboratory, there may be more procedures in between the basics such as semen assessment, extension and storage (Bakst and Dymond, 2013).

The aim of artificial insemination is to ensure a series of fertilised eggs in between consecutive inseminations. In other to achieve this, there must be inseminations which help to restock the spermatozoa number in the uterovaginal junction and spermatozoa storage tubules weekly. Avians do not undergo estrous cycle which requires synchronisation to allow animal to ovulate for copulation. On the other hand, approximately 7 to 10 days prior to the first ovulation, hens mate and spermatozoa move up the vagina into the spermatozoa storage tubules. At the beginning of egg production,

each spermatozoon is slowly released from the sperm storage tubules, migrated to the anterior end of the oviduct, and interacts with the outer layer of the ovum (Froman *et al.*, 2011). Whether fertilised or not, over the next 24 to 26 hours the ovum migrates through the oviduct, accumulating the outer perivitelline layer in the infundibulum, the albumen in the magnum, the shell membrane in the isthmus, and the hard shell in the uterus (also referred to as the shell gland) before oviposition. If fertilised, the blastoderm in the first laid egg consists of 40,000 to 60,000 cells in the turkey and 80,000 to 100,000 cells in the chicken (Bakst, 2011).

Semen preservation with the use of the right diluents is an essential factor in the success of artificial insemination in animal reproduction. The reason is that it boosts the quantity of semen, consequently allowing the use of an ejaculate for more insemination. According to Lafalci, *et al.* (2002), large temperature alterations are injurious to quality of semen and under practical situations, instability in temperature can be easier to circumvent at storage temperature higher than 4 ºC.

2.5.2 Significance of Artificial Insemination in Poultry

The use of artificial insemination as a reproductive tool for poultry (basically turkey industry) is widely practiced in Europe and North America. Although, there are a number of challenges which has limited the adoption in chicken industry over the years (large number of birds to be inseminated weekly, increased cost of labour and the initial investment on cages for birds), the merits of its adoption cannot be over emphasized. Some of the merits include:

1. Ability to process semen for extension and storage (cold storage/cryopreservation)

2. increased mating ratio;

3. with reduced number of males required, there would be greater selection pressure on the male traits of economic importance and subsequently greater genetic advancement per generation;

4. improved biosecurity;

5. Eradication of stress on female birds as imposed by the weight of the cock during natural mating

2.6 Semen extension and extenders

The need for semen extension and preservation has become more evident in recent times. Semen extension involves the dilution of high quality semen with a suitable medium. Basically, all extenders serve as a source of nutrients to the spermatozoa as well as a buffering medium to maintain diluents' pH. Extenders must be capable of maintaining the fertilising ability of the spermatozoa for use in multiple inseminations. For an extender to be classified as suitable, it is necessary that it must meet the following criteria:

1. provide nutrients for the spermatozoa;
2. maintain osmotic balance of the medium;
3. enable multiple inseminations from a single ejaculate by increasing the volume of semen;
4. high buffering ability to prevent change of pH;
5. capable of preventing bacterial growth in the medium;
6. prevent death of spermatozoa due to direct contact between extender and spermatozoa or due to cold shock as in the case of freezing and cryopreservation;
7. maintain motility of spermatozoa within the recommended level and
8. it should not affect the visibility of spermatozoa during microscopic examination.

The extenders in existence today are on the increase with new extenders doing either just well or better than the older ones. The first extender was a simple sugar or salt solution intended solely as a diluents to increase the volume of semen. Extenders commonly used in poultry semen extension include Lake Extender (Lake, 1967), Beltsville Poultry Semen Extender (Sexton and Geisen, 1982), Ringer's Solution (Tabatabaei *et al*., 2011), Tris Buffer Extender (Thomas *et al*., 1993) among others.

The main objective for extending semen is to increase the volume of the ejaculate so that a large number of females may be inseminated by a given male semen while maintaining its quality. In natural mating, one ejaculate is used to inseminate one female, whereas through artificial insemination and the extension of semen, one ejaculate may be used for several hundreds of females. Diluents are buffered salt solutions used to extend semen, they maintain the viability of spermatozoa *in vitro*, and maximize the number of hens that can be inseminated. Semen diluents are based on the biochemical composition of chicken and turkey semen (Lake, 1995). According to Bilodeau *et al.* (2001), dilution of semen by a diluents leads to a low level of antioxidants which may partly explain the lower fertility rate of frozen thawed semen in comparison with fresh semen.

Seminal plasma plays a vital role in the activation of spermatozoa and maintenance of liveability in the reproductive tract of hen; it is however harmful to spermatozoa viability outside the body for a long time. In physiological state, spermatozoa are made active by seminal plasma at ejaculation which allows them to swim further than the spot where semen is deposited in the female. Spermatozoa are exposed to seminal plasma for a long time only during *in vitro* storage. Hence, it is traditional to extend the semen with diluents, to supply nutrients for the spermatozoa for the period of storage *in vitro*, to reduce the toxic constituents in the seminal plasma and to cushion the effect of their metabolic waste (buffer). The extension with diluents also allows the semen to be divided into multiple semen doses, each containing a definite number of spermatozoa that has been determined to be sufficient for good fertility in inseminated females (Morrell, 2011).

Addition of various components of extenders to semen maintains motility, fertilising capacity and preserve spermatozoa membrane integrity (Sarlos *et al*., 2002; Riha *et al*., 2006). Glutamic acid, the most prominent amniotic constituent of avian seminal plasma, became a standard component of diluents (Lake and Mc Indoe, 1959). Egg yolk is generally accepted to be an effective agent in semen extenders for protection of spermatozoa against cold shock and the lipid phase transition effect (Aboagla and Terada, 2004). However, the use of chilled stored semen diluted in egg yolk based semen extenders is limited by its relatively short time fertilization capacity and individual

differences in egg yolk due to different period of egg storage (Aurich *et al*., 1997). Metabolic activities of spermatozoa are reduced by both hypertonic and hypotonic extenders, and could affect the integrity of cell membrane leading to the clustering of the spermatozoa (Latif *et al*., 2005). Essential functions general to all extenders include osmolarity, provision of energy for the spermatozoa and the maintenance of pH. The metabolic rate and motility of spermatozoa can therefore be manipulated by decreasing the pH of extender below 6.0. According to Donoghue and Wishart, (2000), a high pH increases the metabolic rate and a low pH reduces the spermatozoa motility of spermatozoa *in vitro*.

2.7 Maintenance of spermatozoa viability *in vitro*

Components of spermatozoa membranes are made up of lipids which are involved in a sequence of processes that eventually affect their fertilising ability. According to Scott, (1973), lipids act as a likely supply of energy at some point in *in vitro* storage under anaerobic conditions most especially in birds and mammals. They are also an essential component of the spermatozoa plasma membrane. They are consequently involved in the different periods of maturation, capacitation, acrosome reaction, a chain of biochemical and functional changes necessary prior to fertilisation (Breque *et al.,* 2003).

The spermatozoa plasma membrane is composed of lipids which from a biochemical standpoint reveal important variation from the membranes of somatic cells. For instance, in animal species like mammals, fish, bird etc, spermatozoa membranes include phospholipids, high concentrations of sterols and also high densities of lipids that are ether-linked (Parks and Lynch, 1992). Avian spermatozoa contains phospholipids which are supplemented largely with n-6 polyunsaturated fatty acids which includes docosatetraenoic acids (22:4n-6) and arachidonic acids (20:4n-6) (Surai *et al*., 1998). The elevated levels of polyunsaturated fatty acids makes them highly susceptible to lipid peroxidation (Surai *et al.,* 2000a), which is itself linked to male sterility (Breque *et al.,* 2003). Consequently, spermatozoa membranes should be protected by an extremely effective antioxidant system which is capable of preventing peroxidative damage all through *in vivo* and *in vitro* storage.

Earlier studies in turkey and chicken discovered that lipid peroxidation of spermatozoa membranes takes place during *in vitro* storage in just a few hours both at body-like temperatures as well as at 0 ^{o}C (Bakst, 1993). According to Surai *et al*. (2000a) buildup of thiobarbituric acid reactive substances which result in intoxicating lipid peroxidation was reported in duck semen.

As reported by Aitken, (1994), so many assessments in mammals have confirmed that the development of peroxides at some stage in semen *in vitro* storage leads to a reduction in spermatozoa capability for spermatozoa–ovum fusion, a decrease in fertilising potential (Auger, 1998) and an alteration in spermatozoa motility (de Lamirande and Gagnon, 1992). With chickens, malondialdehyde production is completely connected with a drastic decline in fertilising potential irrespective of whether motility is maintained or not. Douard *et al.* (2000) reported similar findings in turkey. Usually, malondialdehyde formation during *in vitro* storage of poultry semen is at all times related with a considerable loss of polyunsaturated fatty acids (primarily 22:4 n-6 and 20:4 n-6), a condition comparable to the earlier explaination in mammals.

Damages occur to poultry spermatozoa in spite of the existence of a composite antioxidant system based on a combination of innate antioxidants, which includes glutathione (GSH), vitamin E and vitamin C together with antioxidant enzymes for example superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Surai *et al.,* 2000a). Surai (2002) proposed 3-step stages of protection wherein superoxide dismutase connected by means of glutathione peroxidase and metal-binding proteins stand for the foremost stage in charge of putting a stop to formation free radical. The second level, consisting of natural antioxidants (carotenoıds, uric acid, Vit A, C, and E, glutathione, e.t.c) coupled with glutathione peroxidase hinders and limits peroxides propagation and formation. The last stage is founded on the connection of some enzymes (transferases, proteases, phospholipases e.t.c) in charge of fixing and elimination of cell membrane molecules that have been damaged. In spite of all the well structured planes of protection in opposition to peroxidation, it is worth mentioning that the antioxidant systems

available in poultry semen are incapable of completely preventing the harmful effects of lipid peroxidation throughout extended *in vitro* storage (Breque, 2003).

2.8 Physiological Mechanism for Poultry Spermatozoa Storage *in vivo*

When the spermatozoa enter the spermatozoa storage tubules (SSTs) in the female reproductive tract, poultry spermatozoa sometimes go through short or long time of storage (*in vivo)* in the spermatozoa storage tubules. The duration is dependent on a well organized and effective succession of interactions involving the male gametes and their host glands through which spermatozoa may remain viable and retain their potential to fertilise for weeks or days (Breque *et al.,* 2003).

The report of Fujii (1963) explained the availability of changing amount of lipids in the cytoplasm of spermatozoa storage tubules cell which is dependent on the oviductal functional state. Even though, lipids are reported in all species studied (turkey, duck, chicken), they were more particularly considered in chicken hens. The lipids discovered in the cytoplasm of chickens were recognized as complex lipids or as cholesterol esters commonly dispersed inside the infra and supra nuclear sections of the cytoplasm (Gilbert *et al*., 1968). Their precise function has not yet been well-known. It is theorized that they contribute to the preservation of the spermatozoa membrane integrity (Renden *et al*., 1981). It was also hypothesized to function for residing spermatozoa's metabolic substrate (Bakst, 1987). However, the cytoplasm of spermatozoa storage tubules cell in chicken hens' cells was discovered to be rich in glycogen but not present in turkeys (Bakst, 1987). This glycogen is suspected to serve as energy source while the spermatozoa are stored in the tubules (Breque *et al.,* 2003).

Overall, spermatozoa storage tubules can be viewed as specific membranes retaining a tolerable condition which preserve the functional state and viability of spermatozoa inside the hen's reproductive tract (Bakst *et al.,* 1994). Figure 3 shows spermatozoa storage sites in the oviduct of chicken hen according to Breque *et al,* (2003).

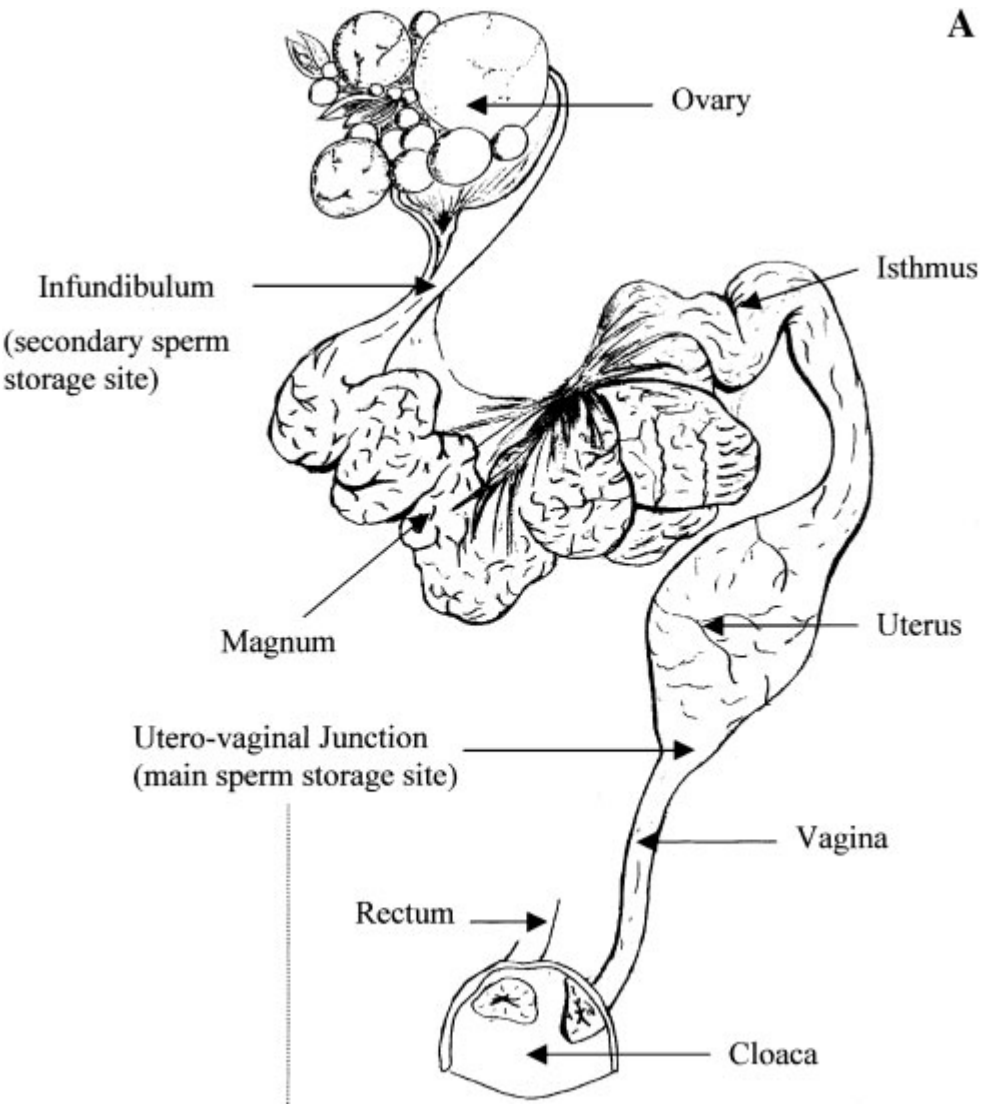

Figure 3: Diagram of the oviduct of a matured chicken hen

Source: Breque *et al.*, (2003)

Despite the fact that an accurate explanation of spermatozoa metabolism in *in vivo* storage is yet to be established, researches conducted over the years have highlighted some approach into the ways poultry spermatozoa used to retain structural integrity all through *in vitro* storage. Also, logical bases now exist to give details on how the immediate surroundings provide by the spermatozoa storage tubule lumen can participate actively in averting peroxidation of structural lipid components (Breque *et al.,* 2003) as explained below.

2.9 Maintenance of spermatozoa viability and functional state in female reproductive tract

Polyunsaturated fatty acids are highly susceptibility to peroxidation which is the most important limiting issue with *in vitro* spermatozoa storage in poultry. Therefore, there is need to know how spermatozoa may strongly defend against the undesirable consequences of prolonged spermatozoa storage in the female tract, since the storage will possibly last up to some weeks under temperature conditions extremely conducive for peroxide formation (39 to 42 ^{o}C based on phase of the ovulatory cycle and animal species). It has been reported earlier that after ejaculation, antioxidant activity of seminal plasma initially protects spermatozoa membrane from peroxidation (Surai *et al.,* 2000a). On the other hand, seminal plasma is quickly eradicated and substituted with the fluid produced by the oviduct, an environment in which biochemical and chemical composition are modified at some stage in the ovulatory cycle (Breque *et al.,* 2003). Reports on chicken have confirmed that, after deposition of semen in the oviduct, spermatozoa may stay in the luminal region of the vagina up to 2–3 days (turkey) or 1 day (chicken) before getting to the storage sites situated in the utero–vaginal junction. It is therefore necessary to maintain the integrity of the membrane all through the prolonged periods prior to fertilisation. Earlier explanations have unveiled the existence of a composite mechanism wherein important antioxidants for example glutathione peroxidase, superoxide dismutase, vitamins C and E, and GSH are activated. Also, the enzymatic activity of the antioxidant is more active in the spermatozoa storage tubules

than in the liver. This is a strong signal that the coordination is particularly tailored to function as innate defense against spermatozoa peroxidation (Breque *et al.,* 2003).

2.10 Physiology of Reactive Oxygen Species

According to Agarwal *et al*., (2003), poor spermatozoa quality has been reported to be associated with high reactive oxygen species (ROS) production in consequence of lipid peroxidation in semen. Reactive oxygen species may have useful or damaging effects depending on the location, time of exposure, concentration and type (Agarwal and Saleh, 2002). In the female reproductive tract, proper motility is not enough to guarantee fertilisation, because spermatozoa have to go via a physiological process called capacitation (Khan, 2011). According to Agarwal *et al*. (2003) spermatozoa generate little quantity of reactive oxygen species like superoxide anion under physiological condition. This superoxide anion efficiently helps in the acrosomal effect and capacitation.

The cell membrane and spermatozoa cytoplasm is more susceptible to the damages done by reactive oxygen species when the quantity of polyunsaturated fatty acids increases (Alvarez and Storey, 1995 as cited in Khan, 2011). For this reason, high production of reactive oxygen species is one of the fundamental causes of sterility (Khan, 2011). The quality of spermatozoa in the original semen aliquot as reported Gomez *et al*. (1998) is inversely proportional to the quantity of reactive oxygen species produced by spermatozoa. Misro *et al*. (2004) reported that hydrogen peroxide is one of the main reactive oxygen species manufactured by the spermatozoon through which intracellular adenosine triphosphate causes its motility restriction and consequent decline in the axonemal proteins. Lipid peroxidation is also cause by elevated quantity of hydrogen peroxide in semen and it eventually leads to cellular apoptosis. While going through differentiation at some points in their finishing stage of maturation, the cytoplasm of most spermatozoa is lost. This cytoplasm is changed into cytoplasmic droplet under some situations. Those spermatozoa are assumed not to be fully formed, able to produce high concentration of reactive oxygen species and functionally faulty (Agarwal *et al*., 2003).

Mitochondrion has been reported to contribute to the production of reactive oxygen species by means of nicotinamide adenine dinucleotide (NADH)-dependent oxido-reductase systems (Hallak *et al*., 2001). The spermatozoa contain sufficient mitochondrion which is responsible for supplying energy needed for motility. The production of reactive oxygen species is appreciably increased in injured mitochondria, which also have a negative effect on mitochondrial efficiency in spermatozoa (Khan, 2011). Wang *et al*. (2003) stated that the potential of mitochondrial membrane is reduced in the spermatozoa of sterile animals with elevated amount of reactive oxygen species produced and it is completely associated with concentration of spermatozoa. By implication, internal mitochondrial membrane potential is highly imperative in the evaluation of spermatozoa function.

Mitochondria function is very important in the process of cell death. Elevated levels of reactive oxygen species dislocate the inner and outer mitochondrial layer, letting loose of activating caspase enzymes and cytochrome C protein which causes cell death (Wang *et al*., 2003). High quantity of cytochrome C in seminal fluid indicated considerable mitochondrial damage caused by reactive oxygen species. Cross linking protein and deoxyribonucleic acid damage also affects semen quality (Khan, 2011). This implies that a spermatozoon with dysfunctional DNA will not be able to fertilise an ovum. Consequently, the fertilisation rate reduces when DNA damages increases (Khan, 2011).

2.11 Antioxidants

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage (Kim and Parthasarathy, 1998). Antioxidants are the agents, which break the oxidative chain reaction, thereby, reducing the oxidative stress (Kumar and Mahmood, 2001). Antioxidants, in general, are the compounds and reactions which dispose, scavenge, and suppress the formation of ROS, or oppose their actions (Bansal and Bilaspuri, 2011). Recent studies demonstrate that supplementation of cryopreservation extenders with antioxidants has been shown to provide a cryoprotective effect on bull, ram, goat, boar, canine, and human sperm quality, thus improving semen parameters, for example,

sperm motility, membrane integrity after thawing (Bucak *et al.*, 2010). Vitamin E (antioxidant) may directly quench the free radicals such as peroxyl and alkoxyl (ROO*) generated during ferrous ascorbate-induced LPO, thus it is suggested as major chain breaking antioxidant (Bansal and Bilaspuri, 2009).

2.12 Tocopherol (Vitamin E)

Tocopherol is a fat soluble vitamin. It is composed of eight isoforms, with four tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol) and four tocotrienols (α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol), α- tocopherol being the most potent and abundant isoform in biological systems. The chromatin head group confers the antioxidant activity to α-tocopherols, but the phytyl tail has no influence. Vitamin E halts lipid peroxidation by donating its phenolic hydrogen to the peroxyl radicals forming tocopheroxyl radicals that, despite also being radicals, are unreactive and unable to continue the oxidative chain reaction (Burton and Traber, 1990). Vitamin E was first detected in turkey semen and afterwards in chicken semen. Researchers have revealed that about 88% of vitamin E found in chicken semen is situated in the spermatozoa. According to Surai *et al*. (1997) quantity of vitamin E in chicken semen is generally based on dietary supplementation and it ranges from 0.46 μg/mL (devoid of vitamin E supplementation) to 1.04 μg/mL (by means of vitamin E dietary supplementation at level of 200 mg/kg). Nonetheless, no more enhancements were noticed by increasing the quantity of vitamin E inclusion up to 1000 mg/kg in feed of the same feeding trial.

The report of Lin *et al*. (2005) stated that egg fertility and it's length are mainly the essential economic qualities that male breeder poultry farmers want and they usually easily influenced by ecological factors for example photoperiod and temperature in addition to the quantity of vitamin E consumed by the animal in feed (Eid *et al*., 2006). Considering the structure of vitamin E, it can be grouped as α (alpha), β (beta) and γ (gamma) tocopherol. According to Surai *et al*. (1997), α-tocopherol is regarded as the main vitamin E in spermatozoa. Next to it is γ-tocopherol which amount to about 5-7% of the total vitamin E content. It was reported by Surai *et al*. (2000a) that Vitamin E

efficiently mops up free radicals and acts on them to produce stable ROOH groups$(Tocopherol + R - OO^{*} \rightarrow Tocopherol^{*} + ROOH)$. They also reported that spermatozoa plasma membrane receives natural firmness from vitamin E. A considerable decline in the vulnerability of the semen to lipid peroxidation has been associated with increase in dietary intake of vitamin E (Lin *et al*., 2005). Likewise, reduced vulnerability to Fe^{+2} induced lipid peroxidation has been achieved through increased level of vitamin E content in semen (Surai *et al*., 1997).

Using vitamin E to sustain and improve productivity via dietary supplementation is a usual practice in poultry industry. The outcome however differs based on the quantity and interval of feeding the vitamin E supplemented diets, measurement criteria, age and genetic stocks (Siegel *et al*., 2001). Earlier studies conducted i*n vitro* revealed that vitamin E is a major chain-breaking antioxidant in the spermatozoa membrane. Substantial decline in spermatozoa motility caused by reactive oxygen species have been linked to cryopreservation and thawing procedures and these effects can be successfully combated by inclusion of vitamin E to cryoprotectants (Surai *et al.*, 2000a). According to Gordon (2005), there have been evidences in cattle that certain steps in the cryopreservation of bull semen result in the production of toxic reactive oxygen species; the freeze thawing of bull semen has been shown to cause a strong reduction of spermatozoa intracellular antioxidants. Control of the level of reactive oxygen species to promote spermatozoa motility and survival by adding antioxidants have been successfully tested for preservation of unfrozen semen; adding antioxidants such as α-tocopherol has been implicated to have a protective effect on metabolic activity and the cellular viability of cryopreserved bull spermatozoa. It is believed that the release of toxic substances by dead spermatozoa may seriously affect the fertilising capacity of their companion cells. It may be noted that semen cryopreservation procedures (cooling and thawing) induce capacitation-like changes in spermatozoa. This function is believed to be partly responsible for the reduced fertility of stored spermatozoa (Gordon 2005).

Decline in fertility of cocks due to age have been associated with reduced amount of vitamin E in the testes (Surai *et al*., 1997). According to the report of Lin *et al*. (2005),

native chicken cocks in Taiwan, consuming feed supplemented with vitamin E at an inclusion rate of 40 to 160 mg/kg feed had enhanced spermatozoa motility and viability while exclusion of vitamin E for a prolonged period may negatively affect the birds. From the same report, they also discovered that vitamin E supplementation of 80 and 160 mg/kg feed at 39 weeks of age have a tendency to enhance spermatozoa concentration and plasma testosterone level. Lin *et al*. (2005) recommended that fertility may be enhanced by adding vitamin E at 160 mg/kg in feed of birds at 49 weeks of age and that deficiency of vitamin E over 39 weeks of age will resulted in lower semen quality. The report of Khan (2011) stated that reduced concentration of functional polyunsaturated fatty acids (C20-22) in phospholipids of spermatozoa can be lessened by supplementing vitamin E up to 200 mg/kg of feed, but no additional improvement was observed when the inclusion level was raised to 1000 mg/kg of feed. In the same study, incubation of semen *in* vitro under pro-oxidant conditions resulted in reduced TBARS (thiobarbituric acid reactive substances - an indicator of lipid peroxidation) by approximately 50% with increased vitamin E (1000 mg/kg of feed) supplementation.

Dietary supplementation of selenium has been reported to enhance the function of vitamin E by boosting the activity of glutathione peroxidase thereby reducing vulnerability of spermatozoa to lipid peroxidation (Surai *et al*., 1998). A probable way for limiting lipid peroxidation is through direct addition of vitamin E into the semen extenders. As reported by Donoghue and Donoghue (1997), a reasonable quantity of vitamin E at 80 μg/mL was discovered to enhance the fertilising potential of turkey semen incubated for 24 hours at 24°C. Furthermore, inclusion of vitamin E to the extended turkey semen resulted in higher motility, better liveability, enhanced membrane integrity and reduced lipid peroxidation. Nonetheless, corresponding research established that supplementation of vitamin E through diet was much more significant in limiting lipid peroxidation than direct inclusion into extenders (Surai *et al*., 2000a). The report of Surai *et al*. (1998) stated that vitamin E supplementation through diet reduced considerably the susceptibility of cock testes homogenates to *in vitro* peroxidation.

2.12.1 Effects of Tocopherol on Fertility of Cock Spermatozoa

The fertility of cock spermatozoa is primarily linked to its qualities which include the volume of semen, spermatozoa motility, spermatozoa concentration, spermatozoa viability and the spermatozoa fertilising capacity. These qualities can however be influenced by quite a lot of environmental factors and endocrine disrupting chemicals, which are absorbed into the body via skin contact, respiration or diet (Rengaraj and Hong, 2015). The resultant undesirable effects may be partly limited or prevented by dietary supplementation with some helpful compounds such as antioxidants, vitamins and minerals or by clinical treatments. Linoleic acid is one of the important polyunsaturated fatty acids that cannot be produced *de novo* in vertebrates. Arscott *et al* (1965) reported that male chickens fed with diets low in vitamin E but rich in linoleic acid exhibited low spermatozoa concentration in the semen and impaired fertilising ability. These adverse effects were prevented by feeding diets containing high levels of linoleic acid (7.3%) supplemented with low (4.3 mg/kg feed) and high (166.3 mg/kg feed) amount of vitamin E. Conversely, supplemented diet with large quantity of vitamin E prevented these negative effects. Contrarily, the above diets were not influenced by weight of the testes, hatchability of fertile eggs, and semen volume.

Avian spermatozoa are rich in polyunsaturated fatty acids particularly, docosatetraenoic acid and arachidonic acid (Surai *et al.,* 2001). The high level of polyunsaturated fatty acids made flexibility of membrane possible, which is necessary for spermatozoa motility and spermatozoa-egg fusion (Khan, 2011). Nevertheless, poultry spermatozoa are highly susceptible to reactive oxygen species by reason of their high levels of polyunsaturated fatty acids, thereby resulting in male sterility (Zaniboni, 2006). Hence, high level of antioxidant status in spermatozoa or semen is a precondition for the avoidance of male sterility.

Surai *et al* (1997) conducted an experiment where cocks were fed diets containing 0, 20, 200, or 1000 mg/kg α-tocopheryl acetate for eight weeks, they discovered during the final two weeks that the concentration of vitamin E in spermatozoa and semen had increased

by 100 percent, and the vulnerability of the semen to lipid peroxidation was reduced in cocks fed 200 mg/kg of vitamin E. In a similar experiment conducted by Zanini *et al.* (2003), cocks were offered a basal diet high in soybean/fish oil and with or without supplementation of vitamin E (30, 200, or 400 mg/kg). After 38 weeks, the roosters fed soybean/fish oil gave the lowest total antioxidant status in the semen. On the other hand, the inclusion of vitamin E to the soybean/fish oil diet showed a rise in spermatozoa viability, motility, and semen volume at 38 weeks. According to Zaniboni *et al* (2006) toms were offered a basal diet enhanced with 60 mg/kg of α-tocopheryl acetate between 26 and 39 weeks of age. Also between age 40 and 60 weeks, the toms were offered a basal diet with additional 2% fish oil and 60 mg α-tocopheryl acetate supplementation. From the result obtained, it was noted that the α-tocopherol level in the turkey semen was improved by 100 percent. Biswas *et al* (2009) also fed 30 weeks old cocks basal diet enhanced with 10, 100, or 200 mg/kg dl-α-tocopheryl acetate, the semen evaluation done in the last 3 weeks of the experiment showed that the ratio of abnormal and dead spermatozoa was significantly lower and the fertilising ability was higher in cocks fed vitamin E at 100 mg/kg inclusion level. Furthermore, the vitamin E level of the spermatozoa and semen was significantly higher in cocks fed Vitamin E at 100 mg/kg inclusion level than the cocks fed Vitamin E at 10 mg/kg inclusion level. These studies showed that improved antioxidant content of spermatozoa or semen is determined by increasing the dietary antioxidant level.

2.12.2 Effects of Tocopherol on Fertility in Hen

The fertilisation ability in hens, similar to that of cocks, is important for successful production of offspring that are in good physical shape. More particularly, in avian species the quality of egg, egg production per day, yolk and albumen constituents, egg weight, egg fertility and hatchability are mainly the essential factors that leads to healthy chicks. The ultimate profitability of hens is dictated by the quantity of fertile eggs produced for hatching (Khan, 2011). The nutrients needed for embryo development is sourced principally from albumin and yolk amassed inside the egg. Poultry eggs have considerable quantity of nutrients such as proteins, lipids, carbohydrates, trace elements

and vitamins. They can be reduced or increased in eggs by manipulating the composition of the diet (Rengaraj and Hong, 2015). The proportion of antioxidants and of total lipids, containing the polyunsaturated fatty acids of the linoleic acid series are comparatively constant in eggs of chicken fed with standard diet, although they are affected by manipulations or important changes in dietary nutrient composition (Lin *et al.*, 2004).

2.13 Selenium

Selenium is an essential trace nutrient for humans and animals. Selenium deficiency has been linked to reproductive problems and reduced spermatozoa quality in rats, mice, chickens, pigs, sheep and cattle (Baiomy *et al.*, 2009). Dietary selenium supplementation has been reported to improve reproductive performance in mice, sheep and cattle. This improvement was however dose-dependent, as all of the parameters returned to baseline values during the post treatment period (Vezina *et al.*, 1996). Meanwhile, high selenium intake has been associated with an impaired semen quality (Dimitrove *et al*., 2007). Selenium is an indispensable element which is highly relevant in animal reproduction (Leonhard, 2000; Surai *et al*., 2000a; Barber *et al*., 2005). Selenium being an essential micronutrient in animals has three levels of biological activity:

(1) Trace concentrations are required for normal growth and development

(2) Moderate concentrations can be stored and homeostatic functions maintained

(3) Elevated concentrations can result in toxic effects.

Selenium interacts with several trace elements and these interactions can be additive, antagonistic, or synergistic, and in some cases the interaction was reversed, i.e. antagonism changed to synergism (Eisler, 2000). The antioxidant impact of selenium has been clarified via its effect as constituent of the antioxidant enzyme glutathione peroxidase (GSH-Px) (Eid *et al*., 2006). Selenium is an integral part of glutathione peroxidase, an enzyme which protects cell internal structures against free radicals and is an antioxidant for cellular membrane lipids (Sanchez-Gutierrez *et al*., 2008). Glutathione

peroxidase activity has been reported in the semen of several species including ram, dog, human, goat, chicken and bull (Kamran *et al.*, 2012)

Various types of seleno-proteins contribute to the regulation of several physiological functions part of which includes stability of spermatozoa cell membrane and antioxidant protection. According to Khan (2011), it is well-known that a fraction of glutathione peroxidase is changed to structural part of the spermatozoa from the enzymatic form. The significance of selenium is apparent from the information that semen supplemented with selenium shows improved spermatozoa motility, storage characteristics and the gradual release of phospholipids and total lipids from spermatozoa to seminal plasma when stored (Dimitrove *et al.*, 2007). Furthermore, in roosters underprovided with selenium, production of leydig cells responsible for testosterone and sertoli cells responsible for spermatogenesis reduced drastically (Edens and Sefton, 2002).

A number of researches have investigated the impact of supplementing antioxidant in semen extenders to protect spermatozoa during semen processing and storage (Szczesniak-Fabianczyk *et al.*, 2006). Some of those studies reported a significant positive correlation between the quantity of selenium in spermatozoa quality and seminal plasma (Noack-Filler *et al.*, 1993). Protective effect of selenium supplementation on spermatozoa motility, membrane integrity and spermatozoa viability before and after freezing in $1\mu gmL^{-1}$ and $2\mu gmL^{-1}$ selenium as observed in a study by Kamran *et al.*, (2012) may be justified by the improved antioxidant enzymes signifying that selenium supplementation could enhance the capability of seminal plasma to decrease oxidative stress. Researchers have also proved that selenium is contained in the keratinous outer membrane of spermatozoa mitochondria which by implication means that selenium plays a major role in preserving a good formation of this structure. Several selenoproteins contribute and control the physiological functions such as stability and antioxidants of cell membranes. The importance of selenium is distinct from the fact that addition of selenium confirms the improved storage alongside the reduced discharge of lipids from the spermatozoa all through prolonged storage (Dimitrove *et al.*, 2007). It is evident from

the above reviews that selenium administered to animals, given in the feed or supplemented to the semen *in vitro* has advantageous in addition to protective effects.

Selenium has however been implicated to possess prooxidant ability at high dosage leading to toxicity. The molecular mechanism of selenium toxicity remains unclear but there is an increasing database that shows the pro-oxidant effect of excess selenium, particularly in the form of selenite (Raisbeck, 2000). The prooxidant activity of selenium at certain doses may also account for cellular apoptosis (Nilsonne *et al*., 2006). Spermatozoa function can be damaged by surplus selenium and by selenium deficiency. Toxic effect of selenium could be ascribed to negative impact of elevated microelement concentration on the spermatozoa insert, considered as an active region of male gamete and with the destruction of process, such as cell respiration or physiological oxidation taking place in mitochondria (Hawkes and Turek, 2001).

2.14 Antioxidative Effects of Vitamin E in combination with Selenium

Vitamin E and selenium are sometimes used jointly and these have incredible effect when combined together. They have harmonizing function in safeguarding the cell against free radicals and lipid peroxidation and as a result, on the reproductive wellbeing of animals. This is because selenium increases the need for vitamin E (Gerald and Combs, 1992). The joint effect of these antioxidants makes them important for each other. Feeding of dietary selenium and vitamin E enhanced spermatozoa concentration, spermatozoa motility, spermatozoa maturation, reduction of abnormalities and higher adenosine triphosphate (ATP) concentration in the semen of boars (Guzman *et al.*, 2000).

The use of selenium and vitamin E independently and in combination are good for the decline in free radical production and lipid peroxidation which predisposes cells to maximum risk to cell apoptosis. For the period of freezing and thawing, a lot of spermatozoa are damaged due to metabolic activities. The use of vitamin E and selenium singly and synergistically can enhance the storage potential of semen because of their enormous effect on decreasing the production of reactive oxygen species (Muhammad *et al.*, 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study 1: Effect of Selenium and α-Tocopherol on Quality of Spermatozoa of Extended Cock Semen

3.1.1 Experimental Site

The study was carried out at the Teaching and Research Farm of the University of Ibadan ($7^{o}20^{1}$N, $3^{o}50^{1}$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.

3.1.2 Experimental Animals

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

3.1.3 Extender Preparation

The extender used for the study was Ringer's solution and its composition is shown in Table 2. 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 3.1: 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 water added to the mixture to reach 1000 ml	

Source: Tabatabaei *et al.* (2011)

3.1.4 Semen Collection and Evaluation

Semen samples were harvested from 15 cocks by using the abdominal massage collection method described by Burrows and Quinn (1937). Semen samples collected in Ependorf tubes were evaluated for colour and impurities after which they were pooled together, maintained at 37 ^{o}C and taken to the laboratory for extension and qualitative parameters evaluation.

The volume of the pooled semen was determined and divided into aliquots of ratio 1:10 semen to extender according to 9 and 5 experimental treatments with 3 replicates in a completely randomized design as shown in experiments 1, 2 and 3, 4 respectively.

3.1.5 Experiment 1: Effects of Selenium and α-Tocopherol Inclusion on Spermatozoa Quality of Extended Cock Semen stored in 4 to 6 °C.

Experimental treatment allotment

Treatment 1: Semen + RS (Control)

Treatment 2: Semen + RS with 25µgmL^{-1} of α-tocopherol

Treatment 3: Semen + RS with 50µgmL^{-1} of α-tocopherol

Treatment 4: Semen + RS with 75µgmL^{-1} of α-tocopherol

Treatment 5: Semen + RS with 100µgmL^{-1} of α-tocopherol

Treatment 6: Semen + RS with 25µgmL^{-1} of selenium

Treatment 7: Semen + RS with 50µgmL^{-1} of selenium

Treatment 8: Semen + RS with 75µgmL^{-1} of selenium

Treatment 9: Semen + RS with 100µgmL^{-1} of selenium

* RS= Ringer's Solution

3.1.6 Experiment 2: Effects of Selenium and α-Tocopherol Inclusion on Spermatozoa Quality of Extended Cock Semen stored at 27 to 29 °C

Experimental treatment allotment

Treatment 1: Semen + RS (Control)

Treatment 2: Semen + RS with 25µgmL^{-1} of α-tocopherol

Treatment 3: Semen + RS with 50µgmL^{-1} of α-tocopherol

Treatment 4: Semen + RS with 75µgmL^{-1} of α-tocopherol

Treatment 5: Semen + RS with 100µgmL^{-1} of α-tocopherol

Treatment 6: Semen + RS with 25µgmL^{-1} of selenium

Treatment 7: Semen + RS with 50µgmL^{-1} of selenium

Treatment 8: Semen + RS with 75µgmL^{-1} of selenium

Treatment 9: Semen + RS with 100µgmL^{-1} of selenium

3.1.7 Experiment 3: Evaluation of the Combined Effect of Selenium and Tocopherol on Spermatozoa Quality in Extended Cock Semen stored at 4 to 6 °C

Experimental treatment allotment

Treatment A: Semen + RS (Control)

Treatment B: Semen + RS with 25µgmL^{-1} of α-tocopherol

Treatment C: Semen + RS with 25µgmL^{-1} of selenium

Treatment D: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium

Treatment E: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

3.1.8 Experiment 4: Evaluation of the Combined Effect of Selenium and Tocopherol on Spermatozoa Quality in Extended Cock Semen stored at 27-29 °C

Experimental treatment allotment

Treatment A: Semen + RS (Control)

Treatment B: Semen + RS with 25µgmL^{-1} of α-tocopherol

Treatment C: Semen + RS with 25µgmL^{-1} of selenium

Treatment D: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium

Treatment E: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

Extended semen samples were stored either inside refrigerator (at 4 to 6 °C) as applicable to experiment 1 and 3) or at room temperature (27 to 29 °C) as applicable to experiment 2 and 4) and evaluated every 3 hours for progressive motility, percentage liveability, total antioxidant capacity and lipid peroxidation.

3.1.9 Extended Semen Quality Evaluation

Progressive Spermatozoa Motility: Progressive spermatozoa motility was determined by mixing a drop of freshly collected semen with a drop of sodium citrate, placed on glass slide and microscopic observations were made on a warm stage at 37 °C at X400 magnification prescribed by Hafez and Hafez (2000).

Percentage liveability: This was done by placing a drop of semen on a warm glass slide and one drop of Eosin-Nigrosin stain was added and mixed gently. The mixture was then smeared on a slide with the edge of another clean slide, air dried and viewed under the microscope at magnification X400. The dead spermatozoa cells were observed to absorb the stain while live spermatozoa cells did not. The live spermatozoa cells were counted and considered as a fraction of the total number of spermatozoa cells that were present (Ewuola and Egbunike, 2010).

Total Antioxidant Capacity: This was determined by centrifuging the semen samples at 3000 rpm to decant the seminal fluid from the spermatozoa cells. A standardised solution of Fe–EDTA complex was prepared by reacting with hydrogen peroxide by a Fenton type reaction, leading to the formation of hydroxyl radicals (OH). These reactive oxygen species degraded benzoate, resulting in the release of thiobarbituric acid reactive substances (TBARS). Antioxidants from the added sample of seminal fluid caused suppression of the production of TBARS. This reaction was then measured with spectrophotometer and the inhibition of colour development defined as the antioxidative activity (AOA) according to Koracevic *et al*. (2001). Different solutions prepared were as shown in appendix 1.

Lipid peroxidation: Glacial acid (3 mL) and 1% TBA (3 mL) solutions were added to test tubes appropriately labeled blank and tests. 0.6 mL of distilled water was added to the blank, while 0.6 mL of each treatment serum sample 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 and their supernatants collected. The supernatant from the blank was used to zero the spectrophotometer (preset at 532 nm) before reading the absorbance of the supernatants from the test solutions. The concentration of Malondialdehyde in the serum was then calculated as follows:

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

3.1.10 Statistical Analysis

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

3.2 STUDY 2: EFFECT OF SELENIUM AND α-TOCOPHEROL INCLUSION ON THE FERTILISING ABILITY OF SPERMATOZOA OF EXTENDED COCK SEMEN

3.2.1 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 clean drinking 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 (20 X 17 X 16 inches high, long and wide respectively). All the cages were kept clean and adequate management practices were carried out regularly.

3.2.2 Semen Collection, Processing and Insemination

Semen samples were harvested from 15 cocks by using the abdominal massage collection method described by Burrows and Quinn (1937). The semen collected into Ependorf tubes were evaluated visually for colour and impurities after which they were pooled together. The volume of the pooled semen was determined and divided into aliquots of ratio 1:5 semen to extender according to treatment layout for experiment 1 and 2. The extended semen was maintained at room temperature prior to insemination

Treatment Layout and Design of Experiment 1 (Room temperature)

Treatment A: Semen + RS (Control)

Treatment B: Semen + RS with 25µgmL^{-1} of α-tocopherol

Treatment C: Semen + RS with 25µgmL^{-1} of selenium

Treatment D: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium

Treatment E: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

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, when 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:

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

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

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

Treatment Layout and Design of Experiment 2 (Refrigerated)

Treatment A: Semen + RS (Control)

Treatment B: Semen + RS with 25µgmL^{-1} of α-tocopherol

Treatment C: Semen + RS with 25µgmL^{-1} of selenium

Treatment D: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium

Treatment E: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

The extended semen was stored at 4 to 6 °C for 3 hours after which semen samples were thawed and insemination.

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, when 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).

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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:

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

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

3.2.3 Statistical Analysis

Data obtained were analyzed using the one-way analysis of variance procedure of SAS (2003) and 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.

CHAPTER FOUR

4.0 RESULTS

4.1 Effects of Selenium and α-Tocopherol Inclusion on Spermatozoa Progressive Motility of Extended Cock Semen Stored at 4 to 6 °C

Result of the effects of α-tocopherol and selenium fortified extender on progressive motility of cold-stored (4 to 6 °C) semen are given in Table 4.1. There were no significant differences ($P>0.05$) among the treatment means except at 6, 12, and 15 hours storage periods. Progressive spermatozoa motility at 0 hour ranged between 76.7% in semen extended without antioxidant fortification and 88.3% in semen extended with Ringer's solution fortified with 75µgmL^{-1} α-tocopherol. It was however least (T3: 71.7%) in semen extended with 50µgmL^{-1} α-tocopherol inclusion level and highest (T4: 85.0%) in semen extended with 75µgmL^{-1} α-tocopherol inclusion level at 3 hours storage period. At 6 hours post storage, the mean value was significantly ($P<0.05$) highest (66.7%) in control (semen with Ringer's solution alone), slightly followed by T5 (100µgmL^{-1} inclusion level of α-tocopherol) and T7 (50µgmL^{-1} inclusion level of selenium) with 63.3%, T2: 25µgmL^{-1} inclusion level of α-tocopherol, T6: 25µgmL^{-1} inclusion level of selenium and T9: 100µgmL^{-1} inclusion level of selenium (61.7%), T3: 50µgmL^{-1} inclusion level of α-tocopherol and T8: 75% inclusion level of selenium (60.0%) while T4 (75µgmL^{-1} inclusion of α-tocopherol) had the least value (55.0%). It was observed that progressive spermatozoa motility values decreased with storage period to as low as 0.0% (T9: 100µgmL^{-1} inclusion level of selenium) at 21 hours evaluation period. Motility values considered acceptable (≥55.0%) were recorded at 0 hour to 6 hours and ≤50.0% was observed at 9 hours to 21 hours. Thus, use of α-tocopherol and selenium as

antioxidants in extended cock semen may be effective for only 6 hours under refrigeration (4 to 6 °C).

4.2 Effects of Selenium and α-Tocopherol Inclusion on Spermatozoa Percentage Liveability of Extended Cock Semen Stored at 4 to 6 °C

Results of the effects of α-tocopherol and selenium fortified extender on percentage liveability of cold-stored semen (4 to 6 °C) are shown in Table 4.2. The result showed that fortified antioxidant had significant differences ($P<0.05$) among the treatments means only at 0, 3, 6, 15 and 21 hours post storage. Percentage liveability at 0 hour was significantly higher ($P<0.05$) with 50µgmL^{-1}, 75µgmL^{-1} and 100µgmL^{-1} inclusion levels of α-tocopherol (T3: 94.17%; T4: 94.33% and T5; 94.67% respectively) and least at 100µgmL^{-1} inclusion level of selenium (T9: 88.67%). At 3 hours, the significantly ($P<0.05$) higher value was observed at 50µgmL^{-1} addition of α-tocopherol (T3: 95.67%) while the significantly ($P<0.05$) lower values were recorded at control (T1:88.67%), 25µgmL^{-1} and 50µgmL^{-1} inclusion levels of selenium (T6: 80.83% and T7: 81.50% respectively). At 6 hours post storage, the mean values was significantly ($P<0.05$) highest in semen extended with 25µgmL^{-1} α-tocopherol inclusion level (T2: 88.17%), slightly followed by 100µgmL^{-1} selenium, control, 100µgmL^{-1} α-tocopherol, 25µgmL^{-1} selenium, 50µgmL^{-1} α-tocopherol, 50µgmL^{-1} selenium and 75µgmL^{-1} selenium (inclusion levels T9: 83.50%, T1: 82.33%, T5: 81.83%, T6: 77.33%, T3: 75.33%, T7: 71.83% and T8: 70.83% respectively) while 75µgmL^{-1} α-tocopherol inclusion level (T4: 67.00%) had the least value. Mean values at 9, 12 and 18 hours were not significantly ($P>0.05$) different across treatment. At 15 hours, 75µgmL^{-1} inclusion level of selenium (T8: 72.00%) was significantly ($P<0.05$) highest while 100µgmL^{-1} inclusion level of α-tocopherol (T5: 41.67%) and 50µgmL^{-1} inclusion level of selenium (T7: 37.00%) were least. However, at 21 hours post storage, 25µgmL^{-1} α-tocopherol inclusion level (T2: 66.67%) had the significantly higher ($P<0.05$) mean value while 0.00% was recorded at 25µgmL^{-1} selenium inclusion level (T6). Thus, use of 25µgmL^{-1} of α-tocopherol in preserving extended cock semen liveability may be effective for up to 21 hours.

Table 4.1: Effects of α-Tocopherol and Selenium Fortified Extender on Progressive Spermatozoa Motility (%) at Refrigeration Temperature

Storage period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
0 hour	76.67	83.33	86.67	88.33	83.33	81.67	80.00	85.00	81.67	3.11
3 hours	73.33	75.00	71.67	85.00	73.33	73.33	73.33	76.67	75.00	3.08
6 hours	66.67^{a}	61.67^{ab}	60.00^{bc}	55.00^{c}	63.33^{ab}	61.67^{ab}	63.33^{ab}	60.00^{bc}	61.67^{ab}	1.11
9 hours	50.00	40.00	36.67	36.67	23.33	43.33	40.00	33.33	36.67	4.71
12 hours	33.33^{ab}	20.00^{b}	23.33^{b}	50.00^{a}	23.33^{b}	30.00^{ab}	26.67^{ab}	25.00^{ab}	20.00^{b}	4.52
15 hours	20.00^{b}	16.67^{b}	20.00^{b}	46.67^{a}	23.33^{b}	23.33^{b}	26.67^{b}	20.00^{b}	16.67^{b}	3.51
18 hours	18.33	10.00	25.00	23.33	13.67	20.00	20.00	6.67	13.33	3.48
21 hours	3.33	8.33	3.67	6.67	10.00	3.67	6.67	0.33	0.00	1.90

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

NB:

SEM: Standard Error of Mean; RS: Ringer's Solution; T1: Semen + RS (Control); T2: Semen + RS with 25µgmL^{-1} of α-tocopherol; T3: Semen + RS with 50µgmL^{-1} of α-tocopherol; T4: Semen + RS with 75µgmL^{-1} of α-tocopherol; T5: Semen + RS with 100µgmL^{-1} of α-tocopherol; T6: Semen + RS with 25µgmL^{-1} of selenium; T7: Semen + RS with 50µgmL^{-1} of selenium; T8: Semen + RS with 75µgmL^{-1} of selenium; T9: Semen + RS with 100µgmL^{-1} of selenium

Table 2.2: Effects of α-Tocopherol and Selenium Fortified Extender on Spermatozoa Liveability (%) at Refrigeration Temperature

Storage Period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
0 hour	92.33^{ab}	92.83^{ab}	94.17^{a}	94.33^{a}	94.67^{a}	91.50^{ab}	93.00^{ab}	92.67^{ab}	88.67^{b}	0.91
3 hours	88.67^{b}	93.33^{ab}	95.67^{a}	85.00^{ab}	84.83^{ab}	80.83^{b}	81.50^{b}	86.33^{ab}	82.67^{ab}	2.34
6 hours	82.33^{abc}	88.17^{a}	75.33^{abc}	67.00^{c}	81.83^{abc}	77.33^{abc}	71.83^{bc}	70.83^{bc}	83.50^{ab}	2.80
9 hours	68.33	64.00	61.83	75.67	74.50	70.50	58.33	67.00	63.33	6.10
12 hours	67.50	57.83	64.00	64.83	47.50	72.67	62.17	71.00	68.00	5.15
15 hours	64.67^{abc}	55.00^{abc}	67.50^{ab}	60.50^{abc}	41.67^{bc}	54.17^{abc}	37.00^{c}	72.00^{a}	48.17^{abc}	4.97
18 hours	59.67	74.17	52.33	57.67	66.67	52.67	60.33	70.00	48.50	5.37
21 hours	38.00^{ab}	66.67^{a}	27.67^{ab}	4.33^{b}	20.00^{ab}	0.00^{b}	44.33^{ab}	36.00^{ab}	52.33^{ab}	9.45

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

4.3 Effects of Selenium and α-Tocopherol Inclusion on Total Antioxidant Capacity of Extended Cock Semen Stored at 4 to 6 °C

The results of the antioxidant effect of α-tocopherol and selenium on total antioxidant capacity of cold-stored semen (4 to 6 °C) are shown in Table 4.3. There were significant differences ($P<0.05$) among the treatment mean values. At 0 hour evaluation period, the highest value (2.2 mmol/L) was recorded in T2 (25µgmL^{-1} α-tocopherol inclusion level), followed by 2.16 mmol/L (T1: control) and 2.12 mmol/L in T3 (50µgmL^{-1} α-tocopherol inclusion level). Meanwhile, mean values of 50µgmL^{-1} and 75µgmL^{-1} inclusion levels of α-tocopherol (T3, T4 respectively); 75µgmL^{-1} and 100µgmL^{-1} of selenium addition (T8 and T9 respectively) did not differ significantly ($P>0.05$) but were significantly different ($P<0.05$) from T2 (25µgmL^{-1} inclusion level of α-tocopherol and T7 (50µgmL^{-1} of selenium inclusion). At 3 hour storage period, the mean values in T2, T3, T4, T5, T6, T7 and T8 (25µgmL^{-1} to 100µgmL^{-1} α-tocopherol inclusion levels and 25µgmL^{-1} to 75µgmL^{-1} selenium addition respectively) were not statistically different ($P>0.05$) and were somewhat similar to the values recorded in control (T1) and 100µgmL^{-1} of selenium (T9). At 6 hours post storage, the mean value was as low as 0.2 mmol/L in 75µgmL^{-1} α-tocopherol inclusion level (T4). Therefore, antioxidant capacity in refrigerated extended cock semen reduces drastically after 3 hours post storage indicating efficient utilisation.

4.4 Effects of Selenium and α-Tocopherol Inclusion on Lipid Peroxidation of Extended Cock Semen Stored at 4 to 6 °C

The results of the effects of α-tocopherol and selenium fortified extender on lipid peroxidation of cold-stored semen (4 to 6 °C) are shown in Table 4.4. There was significant difference ($P<0.05$) only at 0 hour post storage. 25µgmL^{-1} selenium inclusion level (T6: 3.24 µM MDA/10^{-6} spermatozoa) was significantly higher ($P<0.05$) than all other treatments with values ranging from 0.85 µM MDA/10^{-6} spermatozoa to 1.51 µM MDA/10^{-6} spermatozoa. At 3 hours, values obtained range from 0.52 µM MDA/10^{-6} spermatozoa in 50µgmL^{-1} selenium addition (T7) to 2.37 µM MDA/10^{-6} spermatozoa in

25µgmL^{-1} addition of selenium (T6) while at 6 hours, values range between 0.39 µM MDA/10^{-6} spermatozoa in T5 (100µgmL^{-1} α-tocopherol inclusion level) to 3.54 µM MDA/10^{-6} spermatozoa in T6 (25µgmL^{-1} addition of selenium).

Table 4.3: Effects of α-Tocopherol and Selenium Fortified Extender on Total Antioxidant Capacity (mmol/L) at Refrigeration Temperature

Storage period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
0 hour	2.16^{ab}	2.20^{a}	2.12^{abc}	2.04^{abc}	1.99^{bc}	2.00^{bc}	1.96^{c}	2.03^{abc}	2.05^{abc}	0.03
3 hours	1.88^{b}	2.07^{ab}	2.17^{ab}	2.16^{ab}	2.24^{ab}	2.17^{ab}	2.13^{ab}	2.48^{ab}	2.73^{a}	0.11
6 hours	0.51^{a}	0.49^{a}	0.55^{a}	0.20^{b}	0.62^{a}	0.49^{a}	0.42^{ab}	0.54^{a}	0.42^{ab}	0.05

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

Table 4.4: Effects of α-Tocopherol and Selenium Fortified Extender on Lipid Peroxidation (µM MDA/l0^{-6} Spermatozoa) at Refrigeration Temperature

Storage period	Treatments									SEM
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
0 hour	0.98^{b}	1.28^{b}	1.24^{b}	1.18^{b}	1.51^{b}	3.24^{a}	0.85^{b}	1.38^{b}	0.98^{b}	2.51
3 hours	0.92	1.24	0.85	1.47	0.85	2.36	0.52	0.98	1.57	2.99
6 hours	1.38	0.66	0.72	1.77	0.39	3.54	2.82	2.29	2.42	5.36

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

`4.5 Effects of α-Tocopherol and selenium Inclusion on Spermatozoa Progressive Motility of Extended Cock Semen stored at 27 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 4.5. 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 25µgmL^{-1} of α-tocopherol (T2), 75µgmL^{-1} and 100µgmL^{-1} of selenium (T8 and T9respectively) to 96.33% in 75µgmL^{-1} of α-tocopherol (T4). At 3 hours, 100µgmL^{-1} of α-tocopherol (T5: 91.67%) was significantly (P<0.05) higher than control (T1: 81.67%) and 50µgmL^{-1} of selenium inclusion level (T7: 78.33%) but similar to other treatments. However, 50µgmL^{-1} of α-tocopherol inclusion level (T3: 88.33%) was also statistically different (P<0.05) from 50µgmL^{-1} of selenium inclusion T7 (78.33%). The values obtained at 6 hours ranged from 63.33% in T1 and T4 (control and 75µgmL^{-1} of α-tocopherol respectively) to 73.33% in T2 (25µgmL^{-1} of α-tocopherol). At 9 hours post storage, all treatments were significantly similar (P>0.05) except for 100µgmL^{-1} of α-tocopherol inclusion level (T5: 16.67%) and 50µgmL^{-1} 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 ^{o}C cannot exceed 6 hours.

4.6 Effects of selenium and α-Tocopherol Inclusion on Spermatozoa Percentage Liveability of Extended Cock Semen stored at 27 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 4.6. 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 25µgmL^{-1} of α-tocopherol level (T2) to 99.17% in 75µgmL^{-1} of α-tocopherol inclusion level (T4). At 3 hours, all mean values were statistically similar (P<0.05) except for T5:100µgmL^{-1} of α-tocopherol inclusion level (94.50%) which was significantly higher (P<0.05) than T1: control (85.17%), T2:

25µgmL^{-1} of α-tocopherol inclusion level (84.67%) and T9: 100µgmL^{-1} of selenium inclusion level (83.17%). At 6 hours, all values obtained were not significantly different (P>0.05) except for 25µgmL^{-1} of α-tocopherol inclusion level (T2: 89.00%) which was significantly higher (P<0.05) than 75µgmL^{-1} 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 50µgmL^{-1} 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.

4.7 Effects of selenium and α-Tocopherol Inclusion on Total Antioxidant Capacity of Extended Cock Semen stored at 27 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 4.7. 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 4.5: 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.

Table 4.6: 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.

Table 4.7: 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.

4.8 Effects of selenium and α-Tocopherol Inclusion on Total Lipid Peroxidation of Extended Cock Semen stored at 27 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 4.8. There were significant differences among treatments at 0 and 6 hours post storage. At 0 hour, 100µgmL^{-1} 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 100µgmL^{-1} of α-tocopherol inclusion level (T5) and 3.80 µM MDA/l0^{-6} Spermatozoa in 100µgmL^{-1} 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 50µgmL^{-1} of selenium inclusion level (T7). The 2.23 µM MDA/l0^{-6} Spermatozoa (T9: 100µgmL^{-1} of selenium inclusion) at 6 hours post storage was significantly higher (P<0.05) than T2, T3, T4, T5 and T6 (25, 50, 75,100µgmL^{-1} of α-tocopherol inclusion and 25µgmL^{-1} of selenium inclusion respectively) but statistically similar (P>0.05) to T1, T7 and T8 (control, 50 and 75µgmL^{-1} of selenium inclusion level).

Table 4.8: 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.

4.9 Evaluation of the Combined Effects of Selenium and α-Tocopherol on Progressive Motility of Extended Cock Semen Stored at 4 to 6 °C

The results of the combined effects of α-tocopherol and selenium fortified extender on progressive motility of cold-stored semen (4 to 6 °C) are shown in Table 4.9. There were no significant differences ($P<0.05$) among the treatments means except at 3, 12 and 15 hours storage periods. Progressive spermatozoa motility at 0 hour ranged between 91.67% (Td) and 95.00% (Ta and Te). At 3 hours post storage, the mean value was significantly ($P<0.05$) highest (95.00%) in Te, similar ($P>0.05$) to Ta and Tc (90.00%) but differ significantly ($P<0.05$) from Tb (85.00%) and Td (76.67%). However, Ta, Tb and Tc do not differ significantly ($P>0.05$) while Tb also do not differ from Td. Mean values observed at 6 hours post storage was lowest at Tc (68.33%) and highest (78.33%) at Ta. It was observed that progressive spermatozoa motility values decreased with storage period to as low as 0.00% (T5) at 18 hours evaluation period. Acceptable values (≥55.0%) were recorded at 0 hour to 6 hours while ≤50.0% was observed at 9 hours to 18 hours. Therefore, use of α-tocopherol and selenium as antioxidants in extended cock semen may be effective only for 6 hours under refrigeration (4 to 6 °C).

4.10 Evaluation of the Combined Effects of Selenium and α-Tocopherol on Percentage Liveability of Extended Cock Semen Stored at 4 to 6 °C

The results of the combined effects of α-tocopherol and selenium fortified extender on percentage liveability of cold-stored semen (4 to 6 °C) are shown in Table 4.10. There are significant differences ($P<0.05$) among treatment means at 3, 12 and 15 hours evaluation periods. The least value (93.67%) was obtained in Td at 0 hour while the highest value was obtained at Tc (98.50%). At 3 hours, Tb (96.17%) and Te (96.50%) were significantly higher ($P<0.05$) than Td (87.00%) although similar to Ta (92.33%) and Tc (91.83%). The values observed at 6 hours post storage range between 82.17% (Td) and 91.83 (Te). At 9 hours, the highest mean value obtained was at Ta (88.00%) while the least value was at Tc (74.00). Ta (83.67%) at 12 hours evaluation period was however higher ($P<0.05$) than Td (55.33%) although similar to Tb (76.33%), Tc (72.33%) and

Table 4.9: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Progressive Spermatozoa Motility (%) at Refrigerated Temperature

Storage Period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5% (T+SE)	Te 25% (T+SE)	
0 hour	95.00	93.33	93.33	91.67	95.00	1.41
3 hours	90.00^{ab}	85.00^{bc}	90.00^{ab}	76.67^{c}	95.00^{a}	2.08
6 hours	78.33	75.00	68.33	71.67	73.33	4.40
9 hours	46.67	46.67	46.67	53.33	43.33	4.32
12 hours	16.67^{bc}	30.00^{ab}	8.33^{c}	33.33^{a}	8.33^{c}	3.92
15 hours	40.00^{a}	30.00^{ab}	15.00^{bc}	11.67^{bc}	5.00^{c}	4.73
18 hours	10.00	16.67	0.00	5.00	0.00	3.79

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

NB:

T=Tocopherol; SE=Selenium; SEM=Standard Error of Mean; Ta: Semen + RS (Control); Tb: Semen + RS with 25µgmL^{-1} of α-tocopherol; Tc: Semen + RS with 25µgmL^{-1} of selenium; Td: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium; Te: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

Te (66.17%). At 15 hours, treatment means were not significantly different (P<0.05) except at Td (56.67%) which was significantly lower. The values observed at 18 hours ranged between 48.33% (Td) and 64.00% (Tb). It was observed that percentage liveability decreases with time and combined effect of α-tocopherol and selenium at 12.5% each in extended cock semen reduced percentage liveability.

4.11 Evaluation of the Combined Effects of Selenium and α-Tocopherol on Total Antioxidant Capacity of Extended Cock Semen Stored at 4 to 6 °C

The results of the synergistic effects of α-tocopherol and selenium fortified extender on total antioxidant capacity of cold-stored semen (4 to 6 °C) are shown in Table 4.11. There was significant difference (P<0.05) among treatment means only at 0 hour. Te (2.31 mmol/L) was significantly higher (P<0.05) than Td (2.01 mmol/L) although similar (P>0.05) to Ta (2.16 mmol/L), Tb (2.20 mmol/L) and Tc (2.12 mmol/L). At 3 hours, the highest mean value obtained was at Ta (1.88 mmol/L) while the least value was at Te (2.43 mmol/L). However, the mean values obtained at 6 hours ranged between 0.37 mmol/L (Td) and 0.80 mmol/L (Te). Thus, level of antioxidant capacity was increased by the combined inclusion level of α-tocopherol and selenium at 25% and reduced at inclusion level of 12.5% of each under refrigerated temperature.

4.12 Evaluation of the Combined Effects of Selenium and α-Tocopherol on Lipid Peroxidation of Extended Cock Semen Stored at 4 to 6 °C

The results of the synergistic effects of α-tocopherol and selenium fortified extender on lipid peroxidation of cold-stored semen (4 to 6 °C) are shown in Table 4.12. There was no significant (P>0.05) difference across the inclusion levels throughout the period of storage. At 0 hour, treatments mean values obtained range from 0.85 µM MDA/$l0^{-6}$ Spermatozoa (Te) to 1.28 µM MDA/$l0^{-6}$ Spermatozoa (Ta). The least value at 3 hours was obtained at Td (0.46 µM MDA/$l0^{-6}$ Spermatozoa) while the highest value was observed at Tb (1.24 µM MDA/$l0^{-6}$ Spermatozoa). At 6 hours, the treatments mean values range between 0.66 µM MDA/$l0^{-6}$ Spermatozoa (Tb) and 1.38 µM MDA/$l0^{-6}$

Spermatozoa (Ta). It was observed that the values obtained in treatments with 25% α-tocopherol and selenium and their combinations had lower values of peroxidation at 6 hours evaluation period despite their higher values at 0 hour.

Table 4.10: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Spermatozoa Percentage Liveability (%) at Refrigerated Temperature

Storage period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5% (T+SE)	Te 25% (T+SE)	
0 hour	97.50	97.33	98.50	93.67	96.17	1.15
3 hours	92.33^{ab}	96.17^{a}	91.83^{ab}	87.00^{b}	96.50^{a}	1.44
6 hours	91.50	89.33	89.33	82.17	91.83	2.64
9 hours	88.00	87.00	74.00	74.67	87.33	6.05
12 hours	83.67^{a}	76.33^{ab}	72.33^{ab}	55.33^{b}	66.17^{ab}	4.86
15 hours	77.00^{a}	77.50^{a}	76.17^{a}	56.67^{b}	76.50^{a}	4.39
18 hours	52.83	64.00	63.83	48.33	55.00	5.88

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

Table 4.11: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Total Antioxidant Capacity (mmol/L) at Refrigerated Temperature

Storage period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5%(T+SE)	Te 25%(T+SE)	
0 hour	2.16^{ab}	2.20^{ab}	2.12^{ab}	2.01^{b}	2.31^{a}	0.06
3 hours	1.88	2.07	2.17	2.24	2.43	0.18
6 hours	0.51	0.49	0.55	0.37	0.80	0.14

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

Table 3.12: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Lipid Peroxidation (µM MDA/l0^{-6} Spermatozoa) at Refrigerated Temperature

Storage period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5% (T+SE)	Te 25% (T+SE)	
0 hour	0.98	1.28	1.24	0.92	0.85	3.19
3 hours	0.92	1.24	0.85	0.46	0.88	2.57
6 hours	1.38	0.66	0.72	1.05	0.85	2.87

4.13 Evaluation of the Synergistic Effects of Selenium and α-Tocopherol on Progressive Motility of Extended (27 to 29 °C) Cock Semen

The results of the synergistic effects of α-tocopherol and selenium fortified extender on progressive motility of stored semen (27 to 29 °C) are shown in Table 4.13. There was no significant difference (P>0.05) among the treatment means across the storage periods up to 6 hours. The highest value obtained at 0 hour is 93.33% in (Tb) while the lowest value was obtained at Te (86.67%). At 3 hours evaluation period, the values obtained range 46.67% in Td to 56.67% in Tb. It was observed that progressive motility decreased with time to 0.00% in Td and Te at 6 hours post storage while the highest value was 3.33% at Tb. Progressive motility decreased below 50.00% after 3 hours of storage under room temperature except for Td which was already 46.67% at 3 hours post storage.

4.14 Evaluation of the Synergistic Effects of Selenium and α-Tocopherol on Percentage Liveability of Extended (27 to 29 °C) Cock Semen

The results of the synergistic effects of α-tocopherol and selenium fortified extender on percentage liveability of stored semen (27 to 29 °C) are shown in Table 4.14. There were no significant differences (P>0.05) among treatment means except at 0 hour. Td (95.50%) was significantly higher (P<0.05) than Tc (91.17%) and Te (91.50%) although not different (P>0.05) from Ta (94.00%) and Tb (94.67%). At 3 hours, values obtained range from 81.17% in Te to 88.00% in Tb while the lowest value obtained at 6 hours was 58.33% in Te and the highest value was 78.67% in Tc. 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 4.13: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Progressive Spermatozoa Motility (%) at Room Temperature

Storage period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5%(T+SE)	Te 25%(T+SE)	
0 hour	90.00	93.33	88.33	90.00	86.67	2.24
3 hours	55.00	56.67	55.00	46.67	50.00	3.42
6 hours	5.00	3.33	1.67	0.00	0.00	1.29

Table 4.14: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Spermatozoa Liveability (%) at Room Temperature

Storage period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5%(T+SE)	Te 25%(T+SE)	
0 hour	94.00^{ab}	94.67^{ab}	91.17^{b}	95.50^{a}	91.50^{b}	0.89
3 hours	84.33	88.00	84.17	87.50	81.17	2.63
6 hours	76.50	68.17	78.67	71.00	58.33	5.88

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

4.15 Evaluation of the Synergistic Effects of Selenium and α-Tocopherol on Total Antioxidant Capacity of Extended (27 to 29 °C) Cock Semen

The results of the synergistic effects of α-tocopherol and selenium fortified extender on total antioxidant capacity of stored semen (27 to 29 °C) are shown in Table 4.15. There was significant differences ($P<0.05$) among treatment means at 0 and 6 hours storage period. Ta (1.96 mmol/L) and Tc (2.71 mmol/L) were significantly higher ($P<0.05$) than Tb (1.06 mmol/L) although similar ($P>0.05$) to Td (1.86 mmol/L) and Te (2.40 mmol/L). At 3 hours, the least value was obtained at Td (1.98 mmol/L) while the highest value was obtained at Tc (2.35 mmol/L). The significantly highest ($P<0.05$) value obtained at 6 hours evaluation period was at Td (3.17 mmol/L). Although similar ($P>0.05$) to Tc (2.07 mmol/L), it differ significantly ($P<0.05$) from Ta (1.94 mmol/L), Tb (1.73 mmol/L) and Te (0.85 mmol/L).

4.16 Evaluation of the Synergistic Effects of Selenium and α-Tocopherol on Lipid Peroxidation of Extended (27 to 29 °C) Cock Semen

The results of the synergistic effects of α-tocopherol and selenium fortified extender on lipid peroxidation of stored semen (27 to 29 °C) are shown in Table 4.16. 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 (Te) and 1.38 mMMDA/l0^{-6} Spermatozoa (Tb). The least value obtained at 3 hours storage period was at Td (0.39 mMMDA/l0^{-6} Spermatozoa) while the highest value was at 1.28 mMMDA/l0^{-6} Spermatozoa (Te). However, at 6 hours, the least value was at Te (0.52 mMMDA/l0^{-6} Spermatozoa) while the highest value was at Td (1.57 mMMDA/l0^{-6} Spermatozoa).

Table 4.15: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Total Antioxidant Capacity (mmol/L) at Room Temperature

Storage Period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5%(T+SE)	Te 25%(T+SE)	
0 hour	1.96^{a}	1.06^{b}	2.71^{a}	1.86^{ab}	2.40^{ab}	0.33
3 hours	2.18	2.25	2.35	1.98	1.99	0.32
6 hours	1.94^{b}	1.73^{b}	2.07^{ab}	3.17^{a}	0.85^{b}	0.28

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

Table 4.16: Synergistic Effects of α-Tocopherol and Selenium Fortified Extender on Lipid Peroxidation (mMMDA/l0^{-6} Spermatozoa) at Room Temperature

Storage period	Treatments					SEM
	Ta Control	Tb (25% T)	Tc (25% SE)	Td 12.5%(T+SE)	Te 25%(T+SE)	
0 hour	0.98	1.38	1.18	1.05	0.59	3.69
3 hours	0.46	0.59	0.79	0.39	1.28	2.05
6 hours	1.44	0.52	0.72	1.57	0.85	3.32

4.17 *In vivo* Evaluation of the Effects of Selenium, α -Tocopherol and their Synergistic Effect in Extended Cock Semen (27-29 °C)

Figure 4.1 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 significant differences ($P<0.05$) in all the parameters evaluated across the treatments. Fertility rate value was highest in Ta (86.50%) and Tc (86.00%) similar ($P>0.05$) to Td (82.00%) but differed significantly ($P<0.05$) from Te (72.25%) which was also significantly different ($P<0.05$) from Tb (57.00%) being the least value. Non fertile eggs were significantly higher ($P<0.05$) in Tb (43.00%) followed by Te (27.75%) and Td (18.00%) and the least ($P<0.05$) was at Ta (13.5%) and Tc (14.00%). Hatchability of set eggs showed that Ta (69.70%), Tc (71.25%), Td (69.50%) and Te (59.00%) were significantly higher ($P<0.05$) than Tb (40.00%). Hatchability of fertile eggs showed that Ta (80.50%), Tc (82.50%) and Te (81.50%) although not different ($P>0.05$) from Td (79.25%) were higher ($P<0.05$) than Tb (69.50%). It was observed that fertility, hatchability of set eggs and hatchability of fertile eggs were higher ($P>0.05$) at Tc (25% inclusion of selenium) and lowest ($P<0.05$) at Tb (25% inclusion of α-tocopherol) compared to the control.

Table 4.17 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.

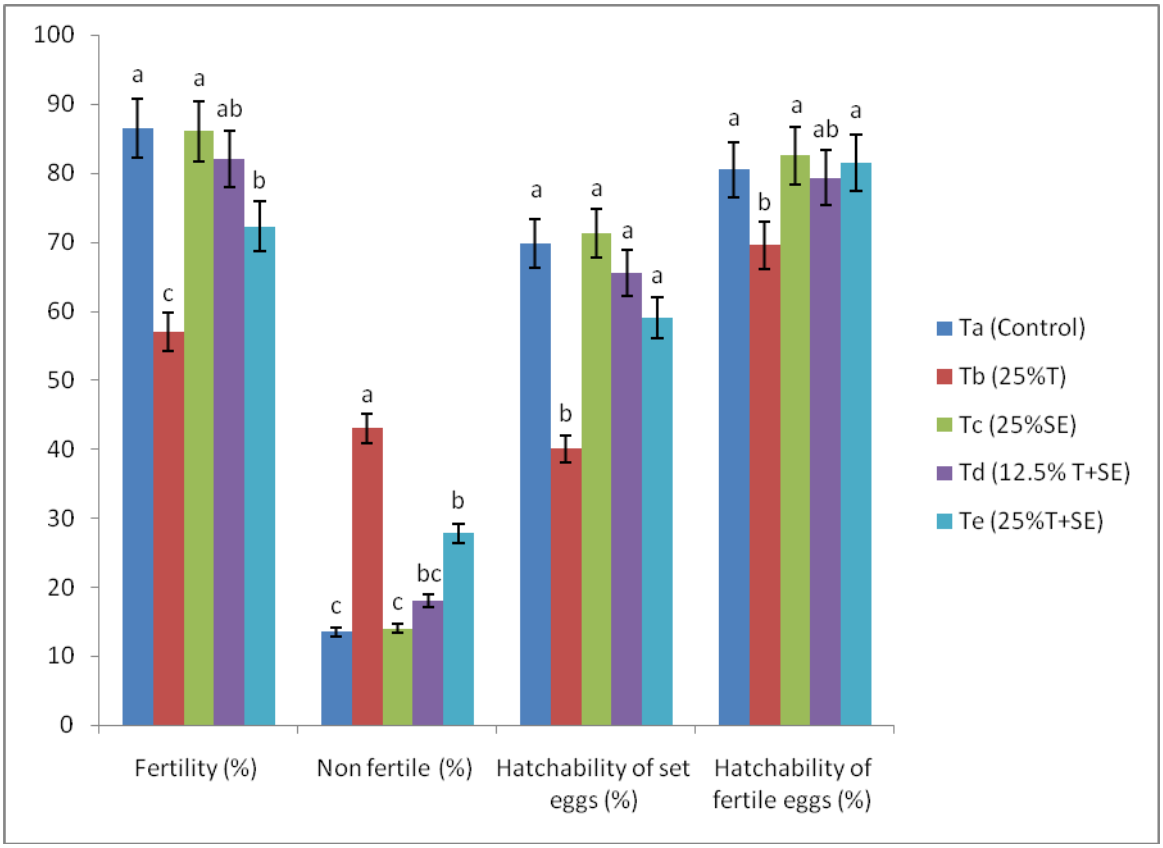

Figure 4.1*: In vivo* Synergistic Effects of α-Tocopherol and Selenium in Extended Cock Semen stored at 27-29 ºC on Fertility and Hatchability in Breeder Hen.

a, b,c: Bars with different superscripts within parameters are significantly different ($P<0.05$).

T=Tocopherol; SE=Selenium; Ta: Semen + RS (Control); Tb: Semen + RS with 25µgmL^{-1} of α-tocopherol; Tc: Semen + RS with 25µgmL^{-1} of selenium; Td: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium; Te: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

Table 4.17: 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 significant correlation at $P < 0.05$

4.18 *In vivo* Evaluation of the Effects of Selenium, α-Tocopherol and their Combined Effect in Extended Cock Semen stored for 3 hours at 4-6 °C

Figure 4.2 presents the effects of α-tocopherol and selenium in extended cock semen cold-stored for 3 hours (4-6 ^{o}C) on fertility and hatchability of eggs in breeder hen. There were significant differences ($P<0.05$) in all the parameters evaluated across the treatments except the hatchability parameters. Fertility rate value was highest in Td (54.25%) and least in Tb (35.25%). Non fertile eggs values range between 45.75% in Td and 64.75% in Tb. Hatchability of set eggs were not significantly different ($P>0.05$) except at Td (49.00%) which was significantly higher ($P<0.05$) than other treatments. Hatchability of fertile eggs showed that Tb (85.25%) and Td (88.50%) although not different ($P>0.05$) from Ta (76.25%) and Te (74.00%) were higher ($P<0.05$) than Tc (55.25%). It was observed that hatchability of set eggs at Td was higher than other treatments including the control. Consequently, inclusion of α-tocopherol and selenium combined at 12.5% of each in cock semen extension stored for 3 hours inside refrigerator (4-6 ^{o}C) may enhance egg fertility and hatchability.

Table 4.18 shows the correlation of fertility parameters of extended cock semen fortified with α-tocopherol and selenium and cold-stored for 3 hours inside refrigerator (4-6 ^{o}C). Positive correlation coefficients were observed at fertile eggs with hatchability of set eggs (55.00%), fertile eggs with hatchability of fertile eggs (43.00%) and hatchability of set eggs with hatchability of fertile eggs (46.00%) while negative correlation coefficients were observed at 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.

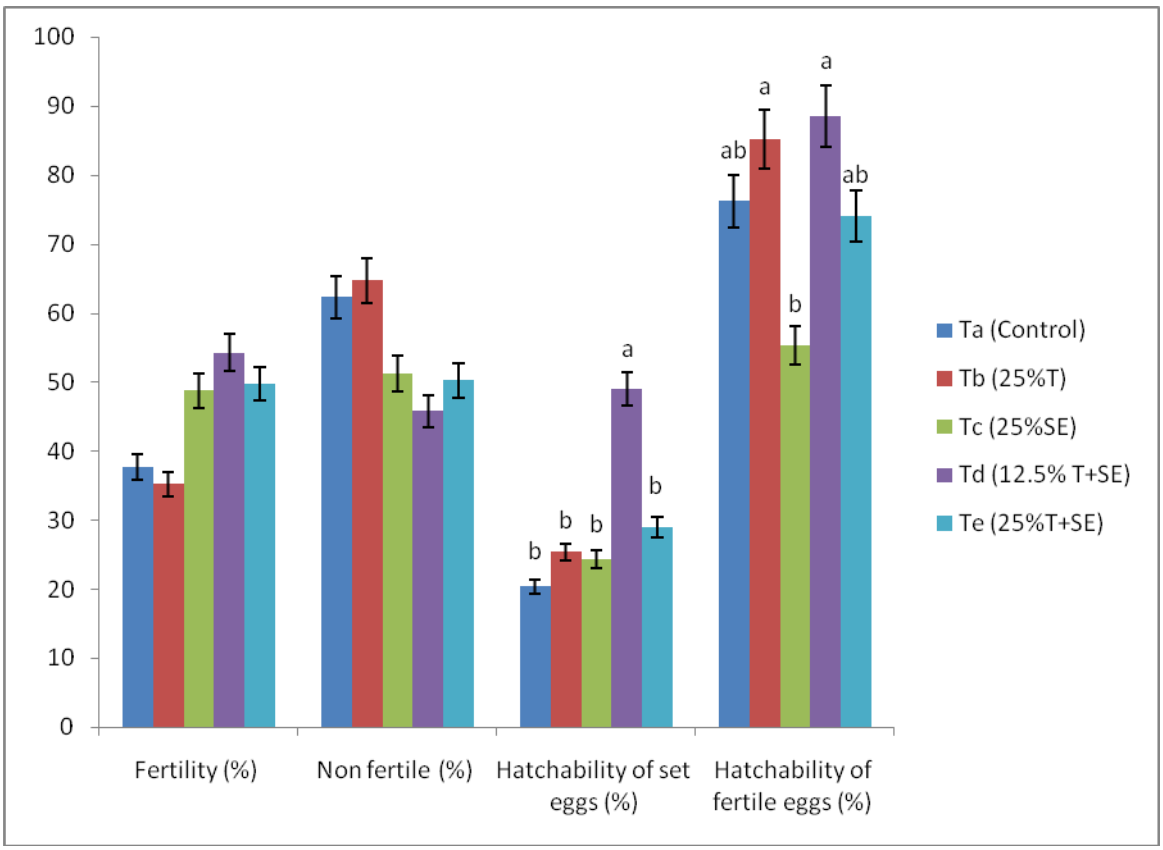

Figure 4.2: *In vivo* Synergistic Effects of α-Tocopherol and Selenium in Extended Cock Semen stored for 3 hours at 4-6 °C

a, b,c: Bars with different superscripts within parameters are significantly different ($P<0.05$).

T=Tocopherol; SE=Selenium; Ta: Semen + RS (Control); Tb: Semen + RS with 25µgmL^{-1} of α-tocopherol; Tc: Semen + RS with 25µgmL^{-1} of selenium; Td: Semen + RS with 12.5µgmL^{-1} each of α-tocopherol and selenium; Te: Semen + RS with 25µgmL^{-1} each of α-tocopherol and selenium

Table 4.18: Correlation of Fertility Parameters of Extended Cock Semen stored for 3 hours at 4-6 ^{o}C

Parameters	Fertile eggs	Non fertile eggs	Hatchability of set eggs	Hatchability of fertile eggs
Fertile eggs (%)	1.00	-1.00*	0.55*	0.43
Non fertile eggs (%)		1.00	-0.55*	-0.43
Hatchability of set eggs (%)			1.00	0.46
Hatchability of fertile eggs (%)				1.00

* There is significant correlation at $P < 0.05$

CHAPTER FIVE

5.0 DISCUSSION

5.1 Effect of Selenium and α-Tocopherol Inclusion on Spermatozoa Quality of Refrigerated Extended Cock Semen

It was observed that progressive motility decreased to 0% at 21 hour post storage and was above 50% up to 6 hours. Motility from 50% and below is not advisable for insemination hence, for this experiment, results from 0 to 6hrs are of utmost importance. This result was within the range reported by Tabatabaei *et al*. (2011) who had a range of 49% - 71% at 6 hours of storage at 4 ^{o}C in extended chicken semen. However, it disagreed with the report of Blesbois *et al.* (1993) who gave a range of 7.6 to 8.0 (using the scale of 0 to 10) which could be translated to 76% to 80% (using a scale of 0 to 100) for refrigerated extender chicken semen samples after 24 hours. The decline below 50% could be attributed to the absence of energy source in the conventional extender used (Ringer's solution) in this experiment. The significant decline in percentage motility at 50 and 75µgmL^{-1} inclusion of tocopherol and 75µgmL^{-1} inclusion of selenium at 6 hours post storage probably suggested a toxicity of the inclusion levels which might be due to over deposition of the antioxidant on the plasma membrane thereby reducing motility. Probably at these dosages, over a long period of storage, certain biochemical metabolites that were deleterious were generated, thus resulting in toxicity. Work with mammalian species has shown an inverse relationship between degree of lipid peroxidation and spermatozoa mobility in the presence of vitamin E. For example, in the boar, vitamin E improved mobility and decreased lipid peroxidation in stored spermatozoa (Cerolini *et al*., 2000), whereas equine spermatozoa motility was not improved and lipid peroxidation was not reduced in the presence of vitamin E (Ball and Vo, 2002). This was explained by

Blesbois *et al.,* (1993) who reported the complexity of the oxidation system because same drug at some doses being an antioxidant became pro-oxidant at higher doses considering the fact that raw semen already contains some level of tocopherol (Khan, 2011). Tafazoli *et al.* (2005) reported that the pro-oxidant activity of tocopherol while Mézes and Balogh (2009) reported that of selenium. They further stated that the exact mechanism of selenium toxicity remains unclear but there were some data about its pro-oxidant effect particularly in the form of selenite. It could also be further explained that the reduced spermatozoa motility observed were due to the reduction in percentage live cells in the same experiment.

The phenomenon of selenium toxicity at 100µgmL^{-1} inclusion level could be responsible for its significantly lower level of percentage liveability at the initial hour. This suggested that higher dosage of selenium inclusion affects liveability in a negative way. This agreed with the report of Nilsonne *et al.* (2006) who reported the pro-oxidant activity of selenium at certain levels could cause cellular apoptosis. However, the effect was not sustainable over a long period as it was no more different from control after 0 hour of storage. At 6 hours, values obtained were similar to that of Tabatabaei *et* al. (2011) who reported 71 ± 2.84 to 90.13 ± 2.03. The 75µgmL^{-1} addition of tocopherol was not as effective as that of 25µgmL^{-1} although different from the control. This could be due to the reasons explained on the pro-oxidant effect on spermatozoa.

The high value of seminal antioxidant contents observed in treatments with Ringer's solution without α-tocopherol and selenium at varying levels under refrigerated condition at 0 hour corroborated Breque *et al.* (2003) who reported that antioxidants are naturally present in fowl semen. Also, it was observed that the values obtained from α-tocopherol based treatment decreased with increasing level of inclusion, but the reverse was the case for selenium based treatments. This could be due to the rate of solubility of the two antioxidants from the binder as Blesbois *et al.* (1993) reported that the α-tocopherol carrier plays an important role in spermatozoa preservation. At 3 hours post storage, the antioxidants were suspected to be fully released from the binder and the inclusion levels were no longer significantly different except at 100µgmL^{-1} inclusion level of selenium

relative to Ringer solution which contained no exogenous antioxidant. At 6 hours storage period, the variation in the inclusion level was also not significant except at 75µgmL^{-1} level of α-tocopherol which could be attributed to the reduced number of live spermatozoa in that particular treatment sample. This suggested that the number of spermatozoa alive affected the rate of antioxidant utilisation and availability in semen sample during storage.

Lipid peroxidation values were observed to be within the values given by Wishart (1984) in an aerobic incubation for 5 hours at 40 ^{o}C. It was also recorded that lipid peroxides by aerobically-stored samples of semen pooled periodically from randomly-chosen male fowls varied. It was revealed that lipid peroxidation was insignificant across the treatments throughout the periods of storage except for 25µgmL^{-1} inclusion level of selenium. This suggested that the level of selenium added was insufficient to prevent peroxidation. However, this cannot be conclusive since Ringer's solution also contained no antioxidant. It could probably be attributed to the presence of agglutination of spermatozoa in the sample. The indifferences observed across the treatment at 3 and 6 hours of storage despite the increased level of exogenous antioxidants suggested that at reduced temperature (4 to 6 ^{o}C), the rate of metabolic activities of the spermatozoa also reduced. This probably influenced the rate of phospholipids production during semen storage. At 6 hours, values of α-tocopherol-based treatments were observed to be lower than that of selenium-based treatments. This could be due to the presence of α-tocopherol being a direct component of fresh semen whereas selenium will be required to be added. Selenium will only be utilized by the activation of an enzyme known as selenium dependent glutathione peroxidase (Se-GSH-Px) similar to what Breque *et al.* (2003) reported that Selenium with or without α-tocopherol stimulates Se-GSH-Px activity in seminal plasma. Higher values of lipid peroxidation is detrimental to spermatozoa surviveability. However, increasing supplemented α-tocopherol and selenium does not correspondingly reduce the rate of peroxidation.

5.2 Effects of Selenium and α-Tocopherol Inclusion on Spermatozoa Quality of Extended Cock Semen at Room Temperature (27 to 29 °C)

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 °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), Maldjian *et al.* (1998) who reported a decline in spermatozoa motility after 6 hours of storage at 25 °C and Oloye *et al.* (2008) who recommended maximum of 6hrs storage period for buck (post diluted) semen at room temperature. According to Surai *et al.* (2000a), 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 SOD and 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 *et al*., 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 *et al.* (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 *et al.* (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 *et al.* (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 (Aitken *et al.*, 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

utilised in peroxidative defence 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 defence 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 for compared to selenium-based treatments and control which further confirms that α-tocopherol has better potential to reduce the rate of peroxidative damage in extended semen sample under room temperature.

From the discussions so far, it can be deduced that cock semen extended with Ringer's solution loose viability after 6 hours post storage. Increased addition of α-tocopherol and selenium beyond 25µgmL^{-1} in Ringer's solution did not give corresponding increase in progressive motility and percentage liveability of spermatozoa. Addition of exogenous antioxidants to Ringer's solution as semen extender had varied effect on total antioxidant capacity and mitigation of lipid peroxidation during storage. This therefore leads to a consideration of the synergistic effect of α-tocopherol and selenium at 25µgmL^{-1} inclusion level. The possibility of synergistic effect was justified by Breque *et al.* (2003) who stated that chicken semen has been reported to contain a complex antioxidant system based on a cocktail of natural antioxidants which do not act singly. The function of α-tocopherol in relation to the presence of selenium dependent glutathione peroxidase (which are both components of avian semen as reported by Eid *et al.*, 2006) *in vitro* could provide a more efficient form of antioxidative defence during spermatozoa storage. Surai *et al.* (1998) also stated that the addition of selenium improves the function of α-

tocopherol by increasing the action of glutathione peroxidase and lowering vulnerability of spermatozoa to lipid peroxidation. Also, glutathione peroxidase (which is selenium dependent) and tocopherol were reported by Eid *et al.* (2006) to act in synergy to protect chicken spermatozoa against oxidative stress.

5.3 Combined Effects of Selenium and α-Tocopherol in Refrigerated (4-6 °C) Extended Cock Semen

It was observed that spermatozoa motility values decreased below 60% after 6 hours of incubation. However, at 3 hours post storage, the significantly higher value obtained at 25µgmL^{-1} inclusion level each of α-tocopherol and selenium relative to the significantly low value obtained at 12.5µgmL^{-1} inclusion level each of α-tocopherol and selenium suggest that selenium in an optimum proportion with α-tocopherol is required to provide both enzymatic and biochemical defense respectively for stored semen. However, the difference was not significant from the treatment containing Ringer' solution alone which suggested that traces of these antioxidants were present in the fresh semen. The significant decline in motility at 25µgmL^{-1} α-tochopherol inclusion level and at 12.5µgmL^{-1} each of tocopherol and selenium suggested that α-tocopherol did not maintain spermatozoa motility as observed in selenium-based treatments under refrigerated condition. This corroborated the work of Blesbois *et al.* (1993) who reported that motility of spermatozoa were not affected by α-tocopherol during storage of fowl semen but negates the findings of Tabatabaei *et al.* (2011) who reported a higher significant difference in α-tocopherol based treatments relative to the control. Almost all other spermatozoa quality assessment trait affected by lipid peroxidation had a resultant effect on progressive motility.

It was observed that the result of liveability follows a trend similar to that of motility. This suggests that percentage motility is a function of percentage liveability. However, low percentage motility does not automatically suggest low percentage liveability. This was responsible for values above 50% in percentage liveability even when motility was observed to be 0%. It could have been affected by other parameters like plasma membrane integrity and percentage normal morphology. At 3 hours, 12.5µgmL^{-1}

inclusion level each of tocopherol and selenium (87%) was significantly lower which could be attributed to insufficient synergistic effect of α-tocopherol and selenium to maintain the survivability of spermatozoa at levels below 25µgmL^{-1}. This corroborated the work of Tabatabaei *et* al (2011) who recommended inclusion level of 20µgmL^{-1} of α-tocopherol inclusion in extenders for better cock semen quality during storage.

Total antioxidant capacity results of the combined effect of α-tocopherol and selenium revealed that there was a significant increase at 25µgmL^{-1} compared to 12.5µgmL^{-1} of the combination (α-tocopherol and selenium) at different levels. However, they were not different from the control. By implication, the potential of the endogenous antioxidant in the semen sample to prevent lipid peroxidation will not be as efficient as that of the preferred dosage (25µgmL^{-1} inclusion in Ringer's solution). Surai, (2000a) recommended the use of an efficient antioxidant in protecting cell membranes against peroxidative damage. Blesbois *et al* (1993) also stated that the efficiency of added antioxidant is dependent on the extent to which it accumulates on the membranes. However, it was observed that the values of the selenium-based treatments initially increased at 3 hours before decreasing at 6 hours post storage. This trend was also observed in all treatments with an exception of 25µgmL^{-1} of α-tocopherol. It reflected variation in the trend of release of the antioxidants from the binder which corroborated the report of Blesbois *et al* (1993) which stated that the antioxidant carrier plays an important role. This could be due to the reduced metabolic activities of the spermatozoa during cold storage resulting in reduced utilisation of the selenium dependent glutathione peroxidise. The reduced values obtained across the treatments at 6 hours confirmed that the antioxidants were being used up in preserving spermatozoa against peroxidation.

Despite the varied inclusion level of antioxidants, lipid peroxidation values were not influenced by the exogenous addition of α-tocopherol and selenium throughout the period of storage. However, they were within the range (0 to 8nmolMDA/10^{9} spermatozoa) specified by Wishart (1984). It also corroborated the work of Long and Krammer (2003) who reported that α-tocopherol did not reduce lipid peroxidation during storage. The maximum value obtained in Ringer's solution (1.88 mmolMDA/10^{6} spermatozoa) at 6

hours in comparison to the 8 nmolMDA/10^9 spermatozoa could be due to the difference in incubation conditions. While Wishart (1984) incubated at 40 ^oC for 5 hours, this experiment was carried out at 4 to 6 ^oC for 6 hours which suggested that refrigeration minimized metabolic activities thereby reducing formation of Malondialdehyde. It was also observed that values obtained from selenium-based treatments were lower than that of α-tocopherol alone and that of the treatments with combination of the antioxidants were more reduced compared to the single antioxidant based treatments except at 6 hours of storage where the reverse was the case. This suggested that α-tocopherol has potential of protecting spermatozoa against peroxidation during prolonged storage of semen sample than selenium. Selenium seemingly acted faster but being enzyme-dependent, it was rapidly used up under cold storage compared to α-tocopherol which is not enzyme based. This was similar to the report of Dimitrove *et al.* (2007) that the importance of selenium is evident from the fact that selenium-supplemented semen has slow release of total lipids and phospholipids from spermatozoa to seminal plasma during storage.

5.4 Combined Effects of Selenium and α-Tocopherol in Extended Cock Semen at Room Temperature (27 to 29 ^oC)

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 significant 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.

5.5 *In-vivo* Evaluation of the Effect of Selenium, α-Tocopherol and their Combinations in Extended Cock Semen at Room Temperature (27 to 29 ^{o}C)

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 significantly 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 highly significant (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.

5.6 *In-vivo* Evaluation of the Effect of Selenium, α-Tocopherol and their Synergy in Refrigerated (4 to 6 ^{o}C) Extended Cock Semen

Fertility and hatchability results of artificially inseminated extended cock semen (refrigerated for 3 hours) were generally low. This was similar to the work of Long and Kramer (2003) who reported maximum fertility of 35% in extended turkey semen refrigerated for 24 hours despite the fortification with α-tocopherol at 10 and 40µgmL^{-1} inclusion levels in some treatments. This could be due to the reduced metabolic activities during the storage period which probably resulted in spermatozoa membrane stress. Chatterjee and Gagnon (2001) reported that cooling during semen storage caused both physical and chemical stress on spermatozoa membranes. However, values obtained for hatchability of set eggs showed that 12.5µgmL^{-1} each of selenium and α-tocopherol was higher than other treatments. This could be due to the cushioned effect of the moderate level of the antioxidant inclusion present in the treatment (12.5µgmL^{-1} each of selenium and α-tocopherol) making it similar to unextended semen. The correlation coefficient of fertility to hatchability of set eggs of refrigerated extended cock semen was also low (55%) which suggested that stored cock semen under 4 to 6 ^{o}C might not retain high productivity of artificially inseminated birds and will not be economically viable to poultry farmers in the poultry industry.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The rate of increasing poultry production in Nigeria is yet to make artificial insemination of extended semen a popular phenomenon among poultry farmers. However, it is pivotal to increased poultry production. Previous researches established that lipid peroxidation caused by activities of free radicals is a major cause of low fertility with stored chicken semen which necessitated the inclusion of antioxidants such as α-tocopherol and selenium as captured in this study. Hence, the assessment of the effect of α-tocopherol and selenium at various inclusion levels (singly and synergistically) on extended cock semen and its influence on spermatozoa quality and egg fertility

Semen samples were collected from fifteen Lohmann breeder cocks aged 30 weeks, pooled and divided into nine groups. Each group was extended with Ringers Solution (RS) without antioxidant (T1), RS with α-tocopherol at 25 (T2), 50 (T3), 75 (T4) or 100µgmL^{-1} (T5) and selenium at 25 (T6), 50 (T7), 75 (T8) or 100µgmL^{-1} (T9). Another set of semen was harvested, pooled and extended with RS (T_A), RS+25µgmL^{-1} α-tocopherol (T_B), RS+25µgmL^{-1} selenium (T_C), RS+12.5µgmL^{-1} α-tocopherol+12.5µgmL^{-1} selenium (T_D), RS+25µgmL^{-1} selenium+25µgmL^{-1} α-tocopherol (T_E) were assessed *in vitro* and *in vivo*. All treatments were evaluated at room temperature (RT: 27-29 ^{o}C) and refrigeration (4-6 ^{o}C) at interval of 3 hours until motility dropped below 50%. 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 also divided into five groups and inseminated with each

of the treatments under RT and another seventy-five under refrigeration 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 $\alpha_{0.05}$.

The following outcomes were observed:

1. Cock semen extended with Ringer's solution stored beyond 3 hours at room temperature (27 to 29 ^{o}C) and up to 6 hours under refrigerated condition (4 to 6 ^{o}C) may not be recommended for insemination to prevent low fertility rate.
2. Increased inclusion of α-tocopherol up to 100µgmL^{-1} in Ringer's solution increased progressive motility and percentage liveability of spermatozoa under room temperature.
3. Addition of exogenous antioxidants to Ringer's solution as semen extender had varied effects on total antioxidant capacity and mitigation of lipid peroxidation during *in vitro* storage.
4. α-tocopherol showed the greatest potential *in vitro*.
5. Selenium based treatments showed greater potential for fertility and hatchability at both storage conditions.
6. Apart from 25µgmL^{-1} α-tocopherol at room temperature (27 - 29 ^{o}C) and 25µgmL^{-1} selenium under refrigerated condition (4 - 6 ^{o}C), all extended semen competed favourably with the control *in vivo*.

6.2 Conclusion

α-tocopherol at 100µgmL^{-1} enhanced cock semen quality, but a combination of α-tocopherol and selenium did not *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. For stored cock semen (refrigeration not exceeding 3 hours), Ringer's solution fortified with 12.5µgmL^{-1} each of α-tocopherol and selenium improved chicken fertility and hatchability even though the fertility rates were generally low compared to non-refrigerated extended cock semen.

6.3 Recommendations

1. The use of semen diluent as a tool of artificial insemination should be well adopted in Nigeria poultry industry.

2. 25µgmL^{-1} selenium should be used to fortify Ringer's solution in cock semen extension (under room temperature) for improved fertility and hatchability.

3. The manipulation of antioxidants either singly or in combination in feed should be further researched.

4. Introduction of energy source into Ringer's solution should be investigated for prolonged semen viability during storage.

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APPENDIX

Different solutions prepared for total antioxidant capacity

(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(NH4)2SO4: 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) H2O2: 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 prepared immediately before use. The sodium phosphate buffer and sodium benzoate were kept in a refrigerator (0–4°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:

	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	0.50	0.50	0.49	0.49
Na-benzoate	0.50	0.50	0.50	0.50	0.50	0.50

Acetic acid	-	1.00	-	1.00	-	1.00
Fe-EDTA	0.20	0.20	0.20	0.20	0.20	0.20
H_2O_2	0.20	0.20	0.20	0.20	0.20	0.20

Incubated for 60 minutes at 37°C, then the following were added:

Acetic acid	1.00	-	1.00	-	1.00	-
TBA	1.00	1.00	1.00	1.00	1.00	1.00

Incubated again for 10 minutes at 100°C (in a boiling water bath), then cool in an ice bath. The samples were 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).