EVALUATION OF THE EFFECT OF VITAMINS C AND E ON SPERMIOGRAM AND MALONDIALDEHYDE CONCENTRATION IN CHILLED SEMEN OF NIGERIAN INDIGENOUS TURKEY TOMS (*Meleagris gallopavo*)

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BY

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MARCH, 2016

DECLARATION

I declare that the work in this dissertation titled "**Evaluation of the effect of Vitamin C and E on Spermiogram and Malondialdehyde Concentration in Chilled Semen of Nigerian Indigenous Turkey Toms (*Meleagris Gallopavo*)**" is original, and has been carried out by me in the Department of Theriogenology and Production, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been presented for another degree or diploma at this or any other Institution previously.

Ochife Wilson ECHEKWU	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This Dissertation titled "**EVALUATION OF THE EFFECT OF VITAMIN C AND E ON SPERMIOGRAM AND MALONDIALDEHYDE CONCENTRATION IN CHILLED SEMEN OF NIGERIAN INDIGENOUS TURKEY TOMS (*Meleagris Gallopavo*)**" by Ochife Wilson ECHEKWU meets the regulations governing the award of the degree of Master of Science in Theriogenology of the Ahmadu Bello University, and is approved for its contribution to scientific knowledge and literary presentation.

Dr. J. S Rwuaan Chairman, Supervisory Committee	_____ Signature	_____ Date
Prof. P. A. Abdu Chairman, Supervisory Committee	_____ Signature	_____ Date
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Prof. Kabir Bala Dean, School of Postgraduate Studies	_____ Signature	_____ Date

DEDICATION

This work is dedicated to my beloved, wonderful parents; Professor C.A Echekwu and Mrs H.E Echekwu, for their love, support, prayers and guidance; and also to my siblings (Daniel, Ruth and Favour) for their encouragement and support during the entire period of this work.

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ABSTRACT

Oxidative stress has been established as a major cause of semen deterioration during *in-vitro* storage. The aim of this study was to evaluate the effects of supplementing the diluents of turkey semen with antioxidant vitamins C, E and their combination on spermiogram and lipid peroxidation during in-vitro storage at 4^0C. Twenty healthy turkey toms aged 37-38 weeks with an average weight of 7±0.3kg were used for this study. The toms were randomly placed into four groups (T0, T1, T2 and T3) of five toms. Semen was collected bi-weekly for 8 weeks from the turkey toms within the groups using abdominal massage method. Following collection, the initial semen characteristics and Malondialdehyde concentration were evaluated. Semen collected from individual turkey toms within each group were pooled, evaluated and extended with egg yolk citrate diluent containing no antioxidant (T0), 4 mg/ml Vitamin C (T1), 8 mg/ml Vitamin E (T2) and 4 mg/ml Vitamin C + 8 mg/ml Vitamin E (T3). The extended semen were packaged into bijju bottles and stored at 4^0C for 48 hours. Semen motility, viability, morphology and Malondialdehyde concentration were assessed at 0, 3, 6, 9, 12, 24 and 48 hours post dilution and storage at 4^0C. The motility and viability of the diluted semen decreased across the group with increase in storage time irrespective of the antioxidants added. T2 gave a better motility (55±2.8 %), viability (41.3± 1.5%) and lower morphological defects (24.3±0.3%) over a 24 hour period as compared to T1(0.0 ± 0.0 %, 19.0 ± 3.4 % and 30.3 ± 0.9%, respectively) and T3 (10.0 ± 5.7%, 25.0 ± 4.0% and 26.7 ± 0.3 %, respectively). The poorest motility, viability and morphological defect results at 24 hours of storage, were obtained in the control (0.0 ± 0.0 %, 10.3 ± 1.2 % and 42.7 ± 2.3%, respectively). Malondialdehyde concentration increased with storage time across the group irrespective of the antioxidants supplemented. However, T2 had a lower Malondialdehyde concentration (2.5 ± 0.2

µl/ml) at 24 hour of storage as compared to T0 (3.1 ± 0.2 µl/ml), T1(2.7 ± 0.2 µl/ml) and T3(2.1 ± 0.2 µl/ml). The result from this study showed that supplementation of the diluents of turkey semen with antioxidants resulted in significant ($p < 0.05$) improvement in spermatozoa motility, viability and a reduced rate of lipid peroxidation compared to the control (T0). Vitamin E performed better than vitamin C and a combination of vitamin C+E in maintaining turkey semen motility (55.0 ± 2.9 %), viability (41.3 ±1.4 %), lower rate of morphological defects (24.2± 0.3 %) and a lower MDA level (2.5±0.2) after 24 hour of storage at 4^0C. It was concluded that the supplementation of diluents of turkey semen with Vitamin E or a combination of vitamin C and E could preserve turkey semen for 24 hours at 4^0C.

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ABBREVIATIONS

Hr	hour
i.e	That is
N/E	Nigrosin/Eosin
Nacl	Sodium Chloride
PBS	Phosphate Buffered Saline
SST	Sperm Storage Tubules
TES	Tris Buffer
PAS	Periodic Acid Schiff
ROS	Reactive Oxygen Species
LH	Luteinizing Hormone
FSH	Follicle Stimulating Hormone
MDA	Malondialdehyde
TBARS	Thiobutyric acid reactive substances

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Artificial insemination (AI) has been a critical component of turkey reproduction since the 1960's and is used exclusively for commercial flock reproduction. The contrast in size of the tom and hens and consequent low fertility of the males after natural mating has resulted in the complete integration of AI in commercial turkey production (Donoghue and Wishart, 2000). Reproductive performance is critical to efficient production in poultry. Therefore, suitable selection criteria for males based on semen characteristics in roosters and toms is very critical (McDaniel *et al.*, 1998). Success in AI requires quality spermatozoa which has the capacity to reach the site of fertilization, fertilize the egg and activate embryonic development.

Short-term liquid storage of turkey semen is of great interest in the management of turkey reproduction due to the extensive use of artificial insemination (Slanina *et al.*, 2012). However, the length of storage of undiluted or extended semen is restricted and presents a significant decrease in spermatozoa viability and fertilizing capacity after 24 hours of storage (Donoghue and Wishart, 2000).

Lipid peroxidation of sperm membrane lipids is a major cause of decreased motility and fertilizing ability of mammalian spermatozoa (Jones and Mann, 1977; Aitken *et al.*, 1989; Aurich *et al*., 1997). Avian semen is characterized by high level of unsaturated fatty acid within its phospholipids (Surai *et al.*, 1998c). This makes their semen susceptible to lipid peroxidation and spermatozoa deterioration during storage (Surai *et al.*, 1997; 1998c).

Oxidative stress occurs when production of potentially destructive reactive oxygen species (ROS) exceeds natural antioxidant defenses resulting in cellular damage (Tremellen, 2008; Bozhedomov *et al.*, 2009). Toxic lipid peroxides are reported to cause impairments of sperm cells and may play a major role in the etiology of infertility in males (Firozeh *et al.*, 2010).

Malondialdehyde (MDA) is one of the final products of lipid peroxidation in seminal plasma (Shang *et al.*, 2004). It is an index of lipid peroxidation which may be a diagnostic tool for the analysis of Infertility (Tavilani *et al*., 2005; 2008). Previous studies have demonstrated a direct link between compromised poultry sperm function after *in-vitro* storage and lipid peroxidation (Fujihara and Howorth, 1978; Cecil and Bakst, 1993).

Avian semen contains natural antioxidants including ascorbic acid, tocopherol, catalase, glutathione peroxidase and superoxide dismutase that help to balance lipid peroxidation and prevent excessive peroxide formation (Alvarez *et al.*, 1987; Beconi *et al.*, 1993). However, the endogenous antioxidant activity of seminal plasma and spermatozoa may not be enough to prevent peroxide damage after extension and *in-vitro* storage thus supplementation with antioxidants could improve the semen shelf life (Donoghue and Donoghue, 1997).

1.2 Statement of Research Problem

Despite the heavy dependence of the turkey industry on AI for commercial production, successful methods for long term *in-vitro* storage of turkey semen are still being developed (Long and Kramert, 2003). Generally, the attention of producers has been integrally glued to chicken with minimal attention paid to other poultry species (Monsi, 1994). Undiluted turkey semen does not have the capacity to remain viable for long

periods outside the reproductive tract (Leighton *et al.*, 1968), in contrast to storage period of about sixteen weeks in the turkey hen following artificial insemination (Christensen and Bagley., 1989). Because of the metabolic requirements and physical composition of spermatozoa and seminal plasma, in-vitro storage of turkey semen is highly susceptible to lipid peroxidation. Improvement of long-term storage procedures for poultry semen could have significant impact on the industry (Donoghue and Wishart, 2000).

Suppression of lipid peroxidation through addition of antioxidant vitamins C and E to the diluents have been achieved with relative success in chicken semen (Al-Daraji, 2002). These antioxidants block the production of ROS or counteract oxygen toxicity. However, there is paucity of information on the effect of different antioxidants and their combinations in preserving semen of turkeys. Also, previous studies on poultry semen in Nigeria were carried out using chickens; hence there is paucity of information on the effects of antioxidants on other poultry species like turkeys.

1.3 Justification

The importance of the poultry industry in Nigeria cannot be over emphasized because of the role it plays in human nutrition and the employment opportunities it creates for her teeming population (Eko, 2009). The potential of local poultry cannot be overlooked considering the huge foreign exchange implication resulting from importation of improved exotic stock and the genotype-environment interaction which leads to considerable loss of fitness of the exotic stock (Oluyemi and Oyenuga, 1971; Ibe, 1990). Nigeria is endowed with an impressive array of domestic poultry species, and they include chickens (160 million), guinea fowls (8.3 million), ducks (1.7 million) and turkeys (0.7 million) (Apantaku *et al.*, 1998).

There is no known discriminatory attitude towards the production and consumption of turkeys and their products in Nigeria but that notwithstanding they are limited in population. Despite the numerous advantages of artificial insemination over natural mating in turkeys, turkey semen does not yet have the ability to survive for long period during *in-vitro* preservation (6-12 hours). It has been reported that the addition of antioxidant vitamins to the semen diluents possibly improved the quality of semen during in-vitro preservation. Other reports showed that supplementing the diluent of poultry semen with more than one antioxidant proved beneficial during in vitro preservation of poultry semen. The effects of different antioxidants have been extensively studied in many species including humans. Vitamin C and E appear to be two of the most extensively studied Vitamins because of their multiple beneficial applications and their availability. However, there is paucity of information comparing the effects of the various antioxidants in the preservation of turkey semen in Nigeria. Hence, a comparative study of antioxidant vitamins C, E and their combination in the preservation of semen of turkeys will be useful in designing an effective artificial insemination and breeding programme in turkeys.

1.4 Aim of the Study

The aim of this study was to evaluate the effects of addition of vitamins C and E to egg-yolk citrate diluents on spermatozoa characteristics and seminal malondialdehyde levels in chilled semen of indiginous turkeys.

1.5 Objectives of the Study

The objectives were to:

- Evaluate the spermiogram of indigenous Nigerian turkeys

- Evaluate the effect of antioxidant vitamins C, E and their combination on spermatozoa motility, viability and morphological defects in chilled turkey semen.
- Evaluate the effect of antioxidant vitamins C, E and their combination on malondialdehyde levels in chilled semen of turkeys.

1.6 Research Questions

- Does vitamin C and E supplementation to the diluent of turkey semen have any effect on semen motility, viability and morphology during *in vitro* storage?
- Does vitamin C and E supplementation to the diluents of turkey semen have any effect on malondialdehyde concentration during *in vitro* preservation of indigenous turkey semen?

CHAPTER TWO

LITERATURE REVIEW

2.1 Artificial Insemination

Artificial insemination is a powerful biotechnological tool which involves semen collection, evaluation, processing, preservation and introduction into the genitalia of an estrus female by use of special equipment to get the female animal pregnant without having any physical contact with the male (Rekwot, 2012). It is a way to enhance reproductive efficiency and production in livestock (Heise, 2011).

Artifiicial insemination is one of the technologies commonly referred to as assisted reproduction technologies (ART). They include *in vitro* fertilization (IVF) where fertilization takes place outside the body (Morrel, 2011); embryo transfer (ET) where embryos that have been derived from either *in vivo* or *in vitro* are transferred to a recipient to establish pregnancy (Morrel, 2011); gamete intrafallopian transfer (GIFT) where spermatozoon are injected into the oviduct to get close to the site of fertilization *in vivo* (Morrel, 2011); estrus synchronization where the female reproductive cycle is manipulated so as to breed them within a short interval (Panhwar, 2011); cryopreservation, where spermatozoa or embryos are cryopreserved in liquid nitrogen for use at a later stage (Morrel, 2011).

2.1.1 History of artificial insemination

The history of artificial insemination came into lime light after 1678 when Leeuwenhoek and Ham were able to view spermatozoa which they called “animalcules” (Leeuwenhoek, 1678). Spalanzani, in 1780 performed his first successful insemination in a dog which whelped three puppies 62 days later (Foote, 2002). Practical application of artifitical insemination was started in Russia in 1899 by Ivanov. He performed AI in

dogs, foxes, rabbits and poultry. He developed semen extenders and trained technicians to select superior stallions and multiply their progency through AI. Ivanov proved the viability of AI in reproduction when he opened the testicle of a dead, snow frozen sheep and found live spermatozoa. The technique became more wide spread in application during the 1940s with the discovery of certain semen preservatives (diluents). Advances in AI practice has increased in horses, beef cattle, sheep, dogs, goats, deer, rabbit and buffalo (Roca *et al*., 2000). It has been used in the conservation of rare or endangered species such as primates, elephants and wild felids and can also be used as a tool to increase conception rates in animals that have fertility problems (Heise, 2011).

Artificial insemination helps prevent the spread of infections or contagious diseases that can be passed on when infected animals are in close contact during mating or share the same environment (Morrel, 2011). It enables breeding between animals in different geographic locations or at different times (even after the males death). With AI breeding can still occur in the event of physical, physiological or behavioural abnormalities. Artificial insemination is a powerful tool when linked to other reproductive biotechnologies such as sperm cryopreservation and sperm sexing (Cseh *et al.*, 2012).

2.1.2 Artificial insemination in Nigeria

The first trials of AI were done in 1945 at the Veterinary Research Institute in Vom, where semen was collected and processed locally using native bulls. In 1952, semen was transported from little Harwood cattle breeding centre, Buckinghamshire, United Kingdom as Fresian chilled semen (Zakari, 1978).

Artificial insemination was carried out by Kano Sate Government using Friesian semen in straws imported from United Kingdom. The semen was used by government farms to produce Bunaji x Friesian and to a limited extent in selected settled Fulani herds. In Oyo State, artificial insemination was carried out with semen of various exotic breeds on N'dama cattle for beef cattle production in Fashola Farm since 1962 (Zakari, 1978).

The National Animal Production Research Institute (NAPRI) had its early origin as "Shika Stock Farm" which was established in 1928 by then Department of Agriculture of the Northern Nigeria. The primary objective of the farm was to produce by selective breeding, the male stock for use as stud bulls by native stock owners. Selection was based on milk yield, body weight and temperament. Subsequently, the AI Unit of the NAPRI was established with the sole responsibility of cross breeding indigenous cattle with exotic semen for genetic improvement (Zakari, 1978).

2.2 Turkeys

The turkey is a large gallinaceous bird of the family meleagridae. They are native of north America and now found in all parts of the world serving as an important source of food and income (Arinze, 2014). They are robust birds farmed mostly for meat and can be raised in an integrated organic environment to maximize profit. The modern domesticated turkey descended from one of six subspecies of wild turkey, *Meleagris Gallopavo* (Hogan, 2008). The other subspecies recognized by biologists are: eastern turkeys (*Meleagris gallopavo silvestris*), Florida (*Meleagris gallopavo Osceola*), Rio Grande (*Meleagris gallopavo intermedia*), Merriamis

(*Meleagris gallopavo meriami*), Gould's (*Meleagris gallopavo Mexicana*) and Southern Mexican (*Meleagris gallopavo gallopavo*). The differences among them is primarily

habitat, but there are minor differences in body size and plumage colouration (Mock, 2002).

2.3 Reproductive System of Turkeys

The avian reproductive system differs significantly from that of mammals and most of the parts, although bear similar names to mammalian organs, have widely different anatomical, histological and physiological features (McDonald, 1996).

2.3.1 Reproductive tract of a turkey tom

The turkey tom's reproductive tract consists of a paired testes, epididymis and ductus deferens. Accessory sex glands (seminal vesicle and bulbo urethral gland) which are present in most mammals, are absent in birds (McDonald, 1996).

2.3.2 The testes

The testes in birds, unlike in most mammals are found within the abdominal cavity. They are paired structures situated on either side left and right of the kidney (McDonald, 1996). They are located deep within the abdominal cavity and are only visible after evisceration of other abdominal organs. The testes are suspended from the dorsal body wall in the midline adjacent to the anterior ends of the kidneys just posterior to the lungs. The testes are surrounded by a fibrous capsule which is composed of connective tissue and contractile fibres (Aire and Ozegbe, 2007). The testes has two major functions: to produce sperm (spermatogenesis) and testosterone (steroidogenesis). Microscopically, the testes consist almost entirely of tubular structure known as seminiferous tubules lined by two types of cell lines, the spermatogonia and the sertoli cells, (McDonald, 1996). Testicular asymmetry like observed in other species have also been reported in turkey toms, with the left testis usually larger than the right testis

(Lake, 1971). Senger in 2003 established an accurate method of determining the quantity of semen produced by a bird by measuring the circumference of the testes. Greater semen output was positively correlated to a larger testicular size.

2.3.3 Epididymis

The epididymis is a structure embedded within connective tissues at the attachment of the testes to the dorsal wall and it consists of three parts; the head (caput), body and tail (cauda). It is very short in comparison to that of mammals. The network of seminiferous tubules unites in the epididymis and the contents flow into and through the ductules ultimately empty into the ductus deferens (McDonald, 1996).

2.3.4 Ductus deferens

The ductus deferens in birds is a major storage organ for sperm. It is an extensive, convoluted tube which runs posteriorly along the midline, parallel to the ureter. In the posterior abdomen, the convolutions of each ductus deferens enlarge greatly in the pelvis, it is straight for a short distance prior to becoming sac-like and ending in the cloaca by an erectile papilla (ejaculatory duct) which projects into the latero-ventral urodeum (mid-region of the cloaca); (McDonald, 1996).

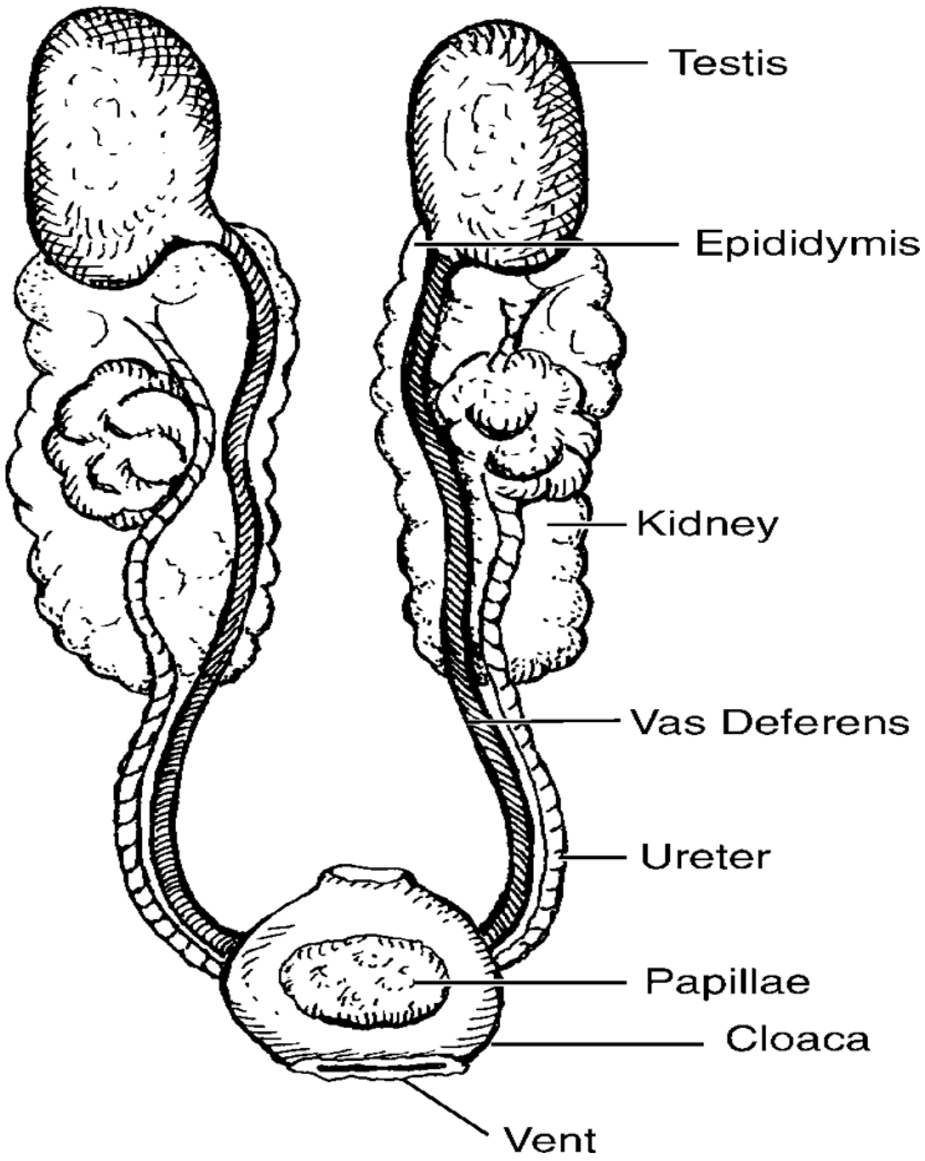


Figure 2.1: The reproductive system of the turkey tom

Source: Banerjee, (2011)

2.4 The Reproductive Tract of the Hen

The female reproductive tract consists of left ovary and oviduct. The ovary develops embryonically as paired structures but after hatching, the right ovary and oviduct degenerates. (McDonald, 1996). The left ovary is found within the body cavity cranial to the left kidney and adjacent to the adrenal glands. It is attached to the body wall by the mesovarian ligament. It is made up of a cluster of developing yolks or ova which are fully formed when a hen is hatched but remain very small until the hen reaches sexual maturity (Jacob and Pescatore, 2013).

2.4.1 The ovary

The ovary consists of two parts, the medulla and cortex. The medulla contains connective tissues, nerves, smooth muscle and blood vessels. The cortex contains the oogonia which are precussor cells from which oocyte and eventually the ovum develops (McDonald, 1996).

2.4.2 The oviduct

The oviduct is attached to the dorsal body wall by the meso-tubarium ligament. The left oviduct is inconspicuous in an immature or non-breeding hen. It is made up of a narrow tube of uniform diameter. In the sexually active bird, the oviduct undergoes tremendous enlargement and occupies a large part of the abdominal cavity. Glandular development results in the thickening of the walls and differentiates it into five functional regions associated with egg formation; the infundibulum, magnum, isthmus, uterus and vagina (McDonald, 1996; Jacob and Pescatore, 2013).

2.4.3 The infundibulum

The infundibulum, also referred to as a “funnel” is the first portion of the oviduct which serves to engulf the ovum released by the ovary. Nonetheless, not all the ovulated oocytes are successfully captured. Some are lost into the body cavity where they are absorbed or become involved in egg yolk peritonitis. The formation of yolk membrane’s other layer; the chalazeferous layer of albumin begins here (McDonald, 1996; Jacob and Pescatore, 2013).

2.4.4 Magnum

This is the longest and most coiled part of the oviduct. It is distinguished from the infundibulum by its greater external diameter and thicker wall caused by the presence of numerous glands packed into massive mucosal folds. The magnum secretes the thick albumin protein around the ovum (McDonald, 1996; Jacob and Pescatore, 2013).

2.4.5 Isthmus

This is a short region with less prominent folds. The name implies a narrow band of tissue connecting two anatomical structures. The inner and outer shell membranes are added in the isthmus (Jacob and Pescatore , 2013).

2.4.6 Uterus

The uterus is of similar diameter as the isthmus but after some distance, expands to form a pouch in which the egg is retained during the entire period of shell formation (McDonald, 1996).

2.4.7 Vagina

The vagina is the short terminal portion of the oviduct proximal to its opening into the urodeum. It does not really play a part in egg formation. Its wall has a well developed muscle and a muscular splinter at the uterine-vagina junction which serves to expel the egg (Jacob and Pescatore, 2013).

2.5 *In vivo* Sperm Storage

In birds following natural mating, sperm are either deposited into the cloaca via "cloacal kiss" in species equipped with a vestigial penis such as chicken and turkeys or introduced into the mid-vagina in species with a retractable penis such as ostrich, ducks and geese (Blesbois and Brillard, 2007). Females of avian species, unlike mammals are endowed with specialized structures located in the oviduct in which sperm can be stored for prolonged period of time. These structures, referred to as sperm storage tubules (SST) provide a specific micro-environment to sustain sperm survival and fertilizing potential (Blesbois and Brillard, 2007). Two sites of sperm storage have been identified in the avian oviduct. One is located at the upper portion of the vagina at the utero vaginal junction (UVJ), and the other at the lower portion of the infundibulum ie, infundibullar SST. Both sites have proved functional but the UVJ and SST are recognized as the main storage sites in poultry as most sperms are stored for a more prolonged period (Briskie and Montgomerie, 1993; Blesbois and Brillard, 2007).

Following mating or artificial insemination, sperm cells are transported to the UVJ by a combination of intrinsic motility and cilia beat activity (Froman *et al.*, 2011; Bakst, *et al.*, 1994). At the UVJ, the sperm are arranged parallel with the head towards the distal end of the sperm storage tubule (SST). Chickens can store sperm in the SST for

up to three weeks, whereas turkeys can maintain sperm for ten weeks in the SST and still lay a fertilized ovum (Christensen and Bagley, 1989; Brillard, 1993). The difference in storage length of sperm in the SST may be related to the number of SST present in the UVJ. Turkeys have been reported to have 20,000-30,000 SSTs, while chickens have been estimated to have about 5,000-13,500 SSTs (Birkhead and Moller, 1992; Bakst and Long, 2010). Increased number of SST is positively correlated with higher fertility (Brillard *et al*., 1998).

The mechanism by which sperm is maintained for prolonged period within the SST remains a puzzle to scientists but findings show that it likely involves the reversible suppression of sperm motility and metabolism, protection and repair of sperm plasma membrane, uptake and storage of molecules to sustain sperm metabolism and maintenance of the SST by removing by-products of sperm metabolism and degraded sperm (Bakst, 1993; Blesbois and Brillard, 2007). A large proportion of sperm plasma membrane is composed of polyunsaturated fatty acids that are highly susceptible to damage induced by lipid peroxidation (Blesbois *et al.*, 1997). The peroxidation of these fatty acids leads to increased damage to the sperm plasma membrane permeability (William *et al*, 1998; Catala, 2012).

A complex system of anti-oxidation enzymes are present in the SST epithelia and presumably interact with luminal sperm to minimize damage due to lipid peroxidation and maintain sperm membrane integrity (Breque *et al*, 2003).

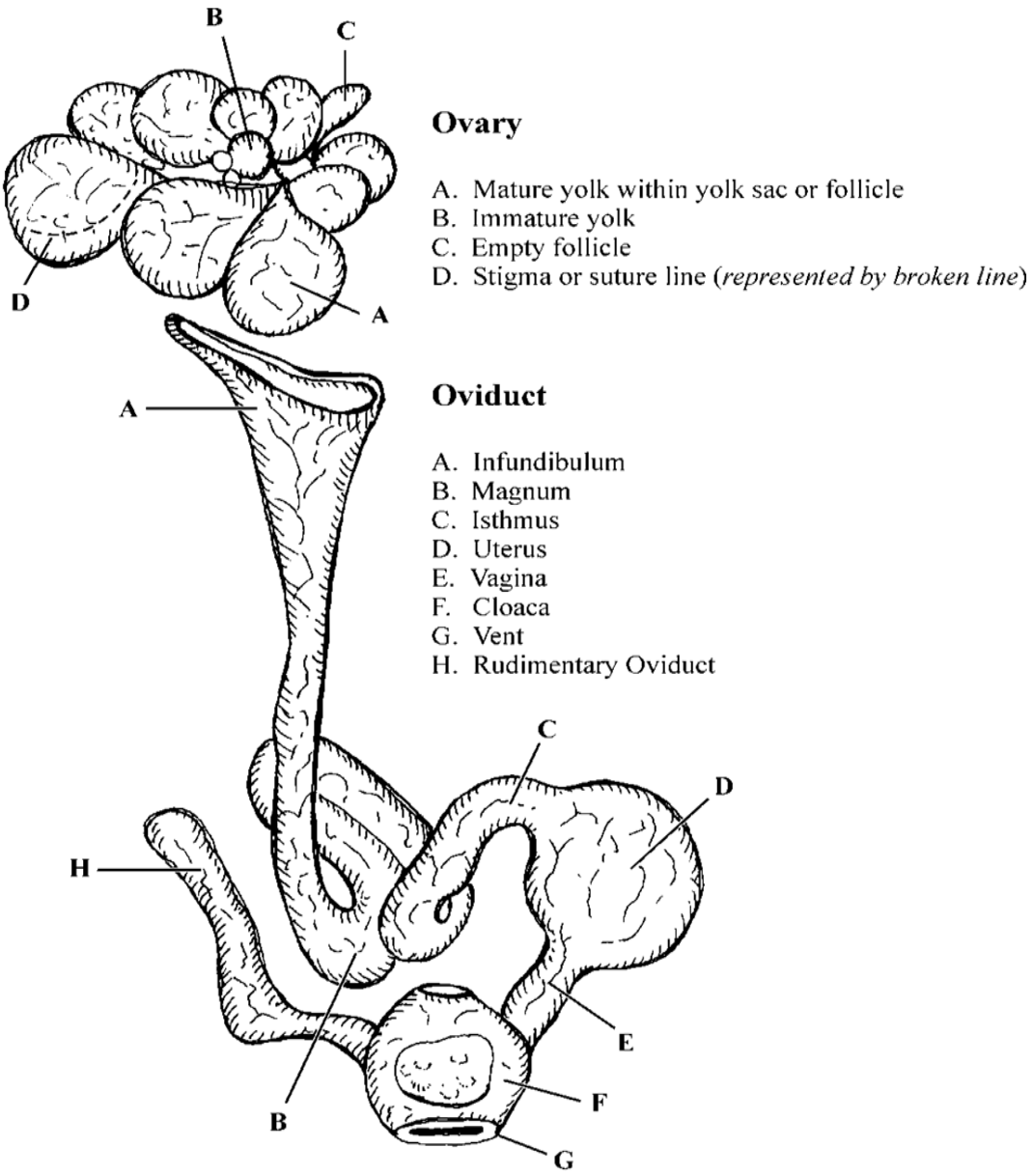


Figure 2.2. Reproductive system of the turkey hen

Source: Banerjee (2011)

2.6 Spermatogenesis

The differentiation of the male germ cell is a complex , highly regulated process that takes place in the seminiferous tubule of the testes (Ventela, 2003). Spermatogenesis is the process of division and differentiation by which spermatozoa are produced in the seminiferous tubules of the testis. The process consist of two phases ie, spermatocytogenesis and spermiogenesis (Gordon, 2005) or three phases ie, spermatocytogenesis, spermatidogenesis and spermeogenesis (Deviche *et al*., 2011). The loose connective tissue linings of the seminiferous tubules possess highly specialized cells, the interstitial cells of leydig which produces male hormone testosterone which plays a major role in signaling and regulation of spermatogenesis (Deutcher, 1996; Walker, 2011).

2.6.1 Spermatocytogenesis

The seminiferous epithelium lining the seminiferous tubule is composed of two cell types; the sertoli cell and the developing germ cell (spermatogonia). The sertoli cells extend from the basement to the lumen and plays a role in supporting spermatogenesis (Bearden *et al*., 2004). The spermatogonia undergoes series of cellular divisions and differentiation beginning from the peripherals and progressing towards the lumen. The spermatocytes are the products of series of division of the spermatogonia. The spermatocytes further undergo meiotic divisions reducing the diploid DNA content to haploid. This process is known as spermatogenesis and the haploid cell resulting from these divisions are known as spermatid (Fishelson *et al.*, 2007).

2.6.2 Spermiogenesis

Spermiogenesis is a metamorphic phase which involves no cell division but rather series of events resulting in the formation of the sperm tail. Changes in the spermatozoa morphology can be seen in the cellular size, shape, nuclear protein, position of the acrosomal granules and localization of the centrioles (Gordon, 2005). The golgi apparatus, an organelle located near the sperm nucleus gives rise to a sub-cellular organelle known as the acrosome. The acrosome develops and forms a cap over the anterior portion of the nucleus two-third of the anterior nucleus (Senger, 2003). The matured spermatid are seen to possesses a flagellum (tail), assembly of mitochondria (mid-piece), the neck piece and complete condensation and shaping of the nucleus (Bearden *et al.,* 2004). The spermatozoon undergoes maturation during transit during which they gain the potential ability to fertilize. This maturation involves several functional changes including development of sustained motility, progressive loss of water and eventual loss of cytoplasmic droplets (Hafez, 1987b). This differentiation of spermatids proceeds through at least four prolonged phases: Golgi, capping, acrosomal, and maturation phases (Ogawa *et al*., 2005; Gordon, 2005). The phases, accomplished through about 16 steps, are useful in identifying specific stages in the cycle of seminiferous epithelium (Hess and Franca, 2008).

Golgi: Golgi apparatus is very important during the early steps of spermiogenesis, as the formation of acrosome depends upon the ability of this organelle to produce vesicles and granules which contain the enzymatic components of the acrosomic system that covers the sperm nucleus (Neves *et al*., 2002; Senger, 2003). Differentiation of the first three steps of round spermatid formation involves a prominent Golgi apparatus that is identified by PAS staining (Ventela *et al*., 2002). Step 1 spermatids have a small, perinuclear Golgi region without acrosomic vesicles or granules (Oatley and Brinster,

2006). Subsequent steps 2-3 show proacrosomal vesicles and granules within the Golgi apparatus, with the formation of a single large acrosomal granule within a larger vesicle that indents on the nucleus.

Capping: This involves steps 4-5 round spermatid, with the acrosomic granules touching the nuclear envelop and the vesicle beginning to flatten into a small cap over the nuclear surface (Hess and Franca, 2008). In steps 6-7 the acrosomic vesicle becomes very thin and the granules flatten. Step 8 is the last round spermatid, and the acrosome flattens over approximately 1/3 of the nuclear surface. Step 8 nuclei begin to change shape (Neves *et al*., 2002).

Acrosomal: Acrosomal steps 9-14 involve migration of the acrosomal system over the ventral surface of the elongating spermatid nucleus. The migration of the acrosome is by around step 14 spermatid and is difficult to identify in typical histological sections (Nakagawa *et al*., 2007).

Maturation: In this stage there are fewer changes in nuclear shape and acrosomal migration (Hess and Franca, 2008). The nucleus continues to condense and the acrosome matures into a thin PAS+ structure that protrudes at the apex but covers nearly all the nucleus, except for the portion connect to the tail (Nakagawa *et al*., 2007). There is removal of excess cytoplasm which results in the formation of prominent cytoplasmic lobes and residual bodies, which contains unused mitochondria, ribosomes, lipids, vesicles and other cytoplasmic components (Nakagawa *et al*., 2007).

Accessory sex glands produce secretions that aid sperm transport and survival. These glands are; prostate gland, cowpers gland and seminal vesicles. Their secretion make up most of the seminal plasma. The largest portion of the seminal fluid is produced by seminal vesicle. (Hafez, 1978b).

2.7 Spermatogenesis and Seminal Epithelial Cycle

Spermatogenesis is a synchronized process by which undifferentiated germ cells are transformed into spermatozoa. The spermatogenic cycle begins with mitotic division of the spermatogonia, proceeds through meiosis and finally with the release of spermatozoa. Cellular associations identified in a cross section of the seminiferous epithelium of the testis has been used to divide the process of spermatogenesis into stages which are species specific (Hess and Paul, 1992). Each stage contains male germ cells at defined phase of differentiation and can be accurately identified by the morphological features of the acrosome and the nucleus of the developing spermatids (Ventela, 2003). A cycle of the seminiferous epithelium is defined as the time required for one sequence through all the various stages, and the number of cycles which occurs from an initial division of a stem spermatogonium to the release of its daughter spermatozoa in to the lumen of the seminiferous tubule is determined by the number of generations associated with a sartoli cell (Clermont,1972). The cellular associations of the various stages of spermatogenesis in mammals have been described and used to further explain the process of spermatogenesis

(Clermont , 1958; Courot *et al* , 1970). Avian species in contrast to mammals do not have a well defined spermatogenic cycle as a result of the existence of atypical stages, for example a number of stages can be seen in one cross section of a seminiferous tubule (6-10 in Japanese quails) and overlapping of germ cells in adjacent stages which makes it difficult to resolve which generations of germ cell are all in one association (Clermont, 1958., Yamamoto *et al*., 1967 and Aire *et al.*, 1980). The difficulty in describing the cellular associations in the avian seminal epithelium is probably due to the rapid transition of certain germ cell cartegories (Lake, 1981). The various stages of spermatogenesis in avian species appear to be of shorter duration than the

corresponding stages in mammals (de Reviers, 1968; Marchand *et al*,1977). For example, while the time duration from the onset of meiosis to the end of spermatogenesis is about 26 days in the mouse (Oakberg, 1956), 29.5 days in the rams (Ortavant, 1956), 37 days in the bull (Hochereau *et al.*, 1964). 14 days in the fowl and drake (de Reviers, 1968; Marchand *et al*., 1977), 11 days in the quail (Amir *et al*, 1963) and 14 days in guinea fowl (Brillard, 1981).

2.8 Endocrine Regulation of Spermatogenesis

In birds, spermatogenesis is regulated by two active pituitary factors, these factors are the gonadotropic hormones, follicle stimilating hormone (FSH) and luteinizing hormone (LH) (Schulz and Muira, 2002). Follicle stimulating hormone and luteinizing hormone are collectively called interstitial cell stimulating hormone in the male and are both secreted by the anterior pituitary (Salisbury *et al*., 1978). Luteinizing hormone plays an important role by stimulating androgen production in the leydig cells, this is through activation of LH receptors. Leydig cells are located between the seminiferous tubules in the interstitial area of the testis, from where the androgens diffuse into the seminiferous tubules mediating the biological activity of LH in spermatogenesis (Schulz and Miura, 2002). Luteinizing hormone acts on the Leydig cells stimulating the production of progesterone which is further converted into the male sex hormone testosterone by androgen converting factor (Senger, 2003). Testosterone is essential for spermatogenesis, for the development of secondary sexual characteristics and for normal mating behavior in males (Bearden *et al.*, 2004). Follicle stimulating hormone directly stimulates FSH receptors on the sertoli cell, which in turn support germ cell survival and spermatogenesis to the secondary spermatocyte stage (Hafez, 2000; Schulz and Miura, 2002).

2.9 Semen Collection

Various techniques of semen collection have been devised and applied to different species of animals. Such methods include the use of artificial vagina (AV), electro ejaculator, rectal massage (digital manipulation) and masturbation (Taylor and Thomas, 2001; Johnson and Prien, 2002; 2003; Johnson *et al*., 2004; Prien and Johnson, 2006). An ideal method of semen collection will be one which is safe for the sire and the collector (Morrel, 2011).

2.9.1 Artificial vagina

The earliest artificial vagina (AV) was invented in 1914 by Giuseppe Amantea, a Professor of Human physiology at the university of Rome, for collecting semen from the male dog. (Taylor and Thomas, 2001). This innovation led other Russian scientists to improve on his idea to create different models suitable for various species such as stallions, bulls and rams (Taylor and Thomas, 2001). Artificial vagina consist of an outer thick, canvas reinforced rubber cylinder about 30 cm long and 6.5 cm in diameter through which is passed an inner liner of latex or rubber which is reflected back at each end and bound firmly to the casing to form a water jacket. Water of the required temperature is introduced through a stopper molded into the outer casing. A rubber cone is attached to one end and graduated glass collecting tube of about 15 ml capacities is inserted into its narrow end. The cone must be securely bound unto the casing and the cone must fit tightly over the mouth of the collecting tube using string or a rubber castrator ring. The artificial vagina is filled with water at 44-46^0 C. Enough water is added to provide a satisfactory pressure on the bulls penis when it is inserted. Lubricant in the form of soft paraffin or petroleum jelly is applied to the mouth of the artificial vagina with a glass rod. A protective cover of sponge material is fitted over the collecting tube and a jacket to cover the tube and cone in order to protect the semen

from cold shock, damage and breakage (Moss *et al.*, 1979). Artificial vagina method of semen collection requires training of the bull or buck to mount the teaser animal or dummy. Artificial vagina uses thermal and mechanical stimulation to stimulate ejaculation (Sekoni, 1990). The test tube containing the ejaculate should be protected from direct sunlight and cold temperatures (Nuti, 2012).

2.9.2 Rectal massage

Rectal massage is the manual massage of the vesicular gland and ampullae of the ductus deferens through the rectal wall (Barth and Oko, 1989). Semen is collected by the gentle massage resulting in stimulation and protrusion of the glans penis from the prepuce (Sekoni, 1990). This method has been associated with contamination of semen with urine (Page, 2002).

2.9.3 Electro- ejaculation

Electro-ejaculation was first adopted in 1952 by Bateelli for collecting semen from guinea pigs. By this method, ejaculation of semen is achieved by inserting a probe or electrode into the male's rectum and stimulating the ampulla and sacral nerve plexus of the reproductive system by gradually increasing voltage in rhythmic fashion with a rheostat for a short period. Successful use requires skills, experience, patience and the knowledge of individual requirement of stimulation (Dorji, 2012). There are currently a number of electro ejaculators in the market of the world, which are either operated only on battery or electric transistorized circuits. This method is used on males of certain species where the use of AV is not practical. It is also used to collect semen from males for AI when the animal is extremely slow in serving the AV or physically incapable of mounting. Electro-ejaculation (EE) is an aversive experience but the level of aversion is probably influenced by the probe type used and the restraint used (Okere *et al.*, 2011).

Modern models of electro-ejaculators consist of a single rectal probe equipped with bipolar electrodes capable of producing ejaculation by electrical stimuli of the vesicular glands, ampulae of the vas deferentia, accessory sex glands, and the hypogastic plexus (Dorji, 2012). Semen harvested by EE has been reported to have a good conception rate as that obtained by other collection methods (Austin *et al.*, 1986).

2.9.4 Abdominal massage

In chickens and turkeys, the abdominal massage technique was developed by Burrows and Quinn in 1937 to collect semen. This technique involves massaging the back and the cloacal region to achieve phallic tumescence. This is followed by a "cloacal stroke", squeezing the region surrounding the sides of the cloaca to express the semen (Bakst and Long, 2010). Additional semen can be expressed after two "cloacal strokes". Additional cloacal strokes may cause damage to the phallic and cloacal regions and contribute to semen contamination (Bakst and Cecil, 1983).

2.10 Factors Affecting Semen Characteristics

2.10.1 Age

Increasing age has been shown to negatively influence reproductive performance of all species. In mammals, deterioration in reproductive performance at the end of the reproductive season partly results from a decrease in male fertility (Elagib *et al*., 2012). Spermatogenesis has been reported to be higher in younger bulls than in older bulls (Kumi-Diaka *et al.*, 1981). Senile bulls have been reported to show low libido, oligospermia and poor sperm quality with resultant zero calf-crop (Rekwot *et al*., 1987). Decrease in fertility and hatchability in poultry are observed when flocks are at the end of the first reproductive period (Kelso *et al*., 1996; Surai *et al*., 2000). The decrease in fertility is associated with a decrease in the number and the viability of spermatozoa and

in motility of spermatozoa in turkey (Bakst and Cecil, 1992). It has also been reported that semen quality of poultry species declined with age as semen from older birds had significantly lower motility, viability and fertility than younger birds (Kotlowska *et al*., 2005).

2.10.2 Season

`Ejaculate characteristics (volume, motility, concentrations, live-dead counts and abnormalities) have been reported to be influenced by seasonal weather conditions such as low temperature and severe winds (Hultnas, 1979; Igboeli and Rakha, 1971; Richardson *et al*., 1977; Sekoni and Gustafson, 1980; Amir *et al*., 1982; Rekwot *et al*., 1987). An experiment carried out in temperate climate showed that fertility of semen frozen in winter was slightly higher than semen frozen in summer (Foote, 1972). An experiment carried out in bulls in the tropical climate revealed a higher percentage of defective spermatozoa in the dry season than in the raining season (Rekwot, 1986). Other studies have also shown that poultry semen motility was lower during the dry and hot season and higher during raining season and with decrease in temperature (Nakarga and Egbunike, 1989). Nayak and Mirsa (1991) observed a decrease in sperm quality with increase in ambient temperature. A significant reduction in sperm concentration of cocks during the summer season was reported by Boone and Huston (1963). Seasonal variation in semen in semen quality of domestic fowl has been documented (Santiago-Moreno *et al*., 2011). McDaniel *et al*. (1996) also reported that an ambient temperature of greater than 31^0C depressed rooster sperm motility, viability and fertilization potential.

2.10.3 Nutrition and management

Nutrition has been shown to have significant effect on semen quality traits. Optimum nutrition is essential to maintain males in good reproductive condition. Starvation on the other hand leads to a marked reduction in semen production which is not permanent but can be reversed once normal feeding is resumed (Boone and Hughes, 1969). Studies on specific nutrient requirement for semen production are rare especially in indigenous poultry species (Obidi *et al*., 2008). During growth of male poultry species dietary protein influence the onset of sexual maturity 9% crude protein and less delays sexual maturity (Surai, 2002). In studies conducted with adult white leghorn cocks fed 9% dietary protein produced normal qualities of semen; and the diet contained 1% calcium and had an energy value of 2853 kcal/kg (Lake, 1971). Ezekwe *et al.* (2003) reported that the effects of underfeeding local cocks on the semen quality traits appear to be more severe on the physical rather than on the biochemical characteristics. This shows that spermatogenic functions of the testes are more responsive to under feeding than the secretory activity of the reproductive tract. Underfeeding diminished the ability of the gonads to respond to gonadotropin stimulation (Davies *et al*., 1957). Moderate reduction of feed intake by 30% to 50% will adversely affect semen production and quality in poultry (Ezekwe *et al*., 2003). The optimal content is a prerequisite not only for a rapid growth but also for normal condition of breeders by influencing the qualitative and quantitative parameters of semen (Sotirov *et al*., 2002). Meyer *et al* (1980) reported that the volume and concentration of spermatozoa ejaculate from exotic turkeys fed with diet containing 12% and 17% protein, respectively, resulted in better semen quality when the higher dietary protein level was used. Bulls fed on high protein ration were shown to have significantly large scrotal circumference, higher body weight and body condition scores and higher sperm output than those on low protein diet

(Rekwot *et al*., 1988). Bulls on high protein diet attained puberty at a significantly earlier age, with significantly higher body weights and also ejaculated first motile spermatozoa earlier than those on low protein diet (Rekwot *et al*., 1987). Gonadal, spermatid and epididymal sperm reserves in bulls on high plane of nutrition were significantly higher than the sperm reserves for both bulls on low and medium planes of nutrition (Asdell, 1955).

2.10.4 Pathological and disease factors

Viral, bacterial, protozoan and other disease conditions associated with the high body temperatures and general loss of body condition have been known to affect spermatogenesis (Roberts, 1971; Rwuaan *et al*., 2012). Testicular hypoplasia, degenerative changes in the seminiferous epithelium and inflammatory changes in the seminiferous epithelium have been associated with defective spermatogenesis (Hafez, 1987b). Parasite infestation have been shown to affect the general state of health and normal physiological processes of turkeys thus affecting spermatogenesis and semen quality.

Leucocytozoon Smithi, a blood parasite decreases the ability of domestic turkeys to mate and decreases productivity in hens (Johnson *et al*., 1938: Byrd, 1959; Hensley and Cain, 1978). Pullorum accused by *Salmonella pullorum* also decreases fertility in domestic turkeys (Hensley and Cain, 1978).

2.11 Semen Evaluation

Semen evaluation is carried out to ensure that the number of functionally normal spermatozoa present in an ejaculate are enough to cause pregnancy and whether the sire has an adequate capacity to produce enough spermatozoa to achieve fertilization (Noakes *et al*., 2001).

The techniques used for assessment of semen quality are based on physical, morphological and biochemical examinations (Ball *et al.*, 1974). Physical tests include: measurement of semen volume, colour, pH, semen concentration and sperm motility (Iversen, 1964).

Morphological examination of spermatozoa for abnormalities or damages due to processing is carried out on stained semen smears using a microscope. Supravital stains are used for the estimation of percentage live and dead spermatozoa (Dott and Foster, 1972).

Biochemical test may be analytic methods which involve the determination of biochemical components of semen such as nucleic acids, enzymes, lipids and cations (Osinowo, 1979). Subjective evaluation of semen has been a method adopted out of necessity because no other alternatives were available. This approach is often prone to observer's bias and error (Nuti, 2012). It has been observed that laboratory tests cannot account for errors which may occur either through handling, dilution and insemination procedures, or through variation in the fertility of the animals inseminated (Melrose, 1962). Significant correlations between conception rates and live/dead counts, motility rating and impendence change frequency have been established (Bishop *et al*., 1954; Cummings, 1954).

Five major criteria exist for evaluating semen. These are: volume, colour, concentration, motility and morphology (Zemjanis, 1970). The greatest output of semen is during the breeding season and is dependent primarily on the species, but can also be dependent on the individual male. For example, a stallions ejaculate is on average 10-20 times greater than that of a bull, while individual bulls have volumes between 2 and 12 ml (Wendee,

2007). In turkey toms, the average volume of the semen is between 0.33 to 0.5 ml. In chickens, the volume is 1 to 2 times that of a turkey (Cynthia and Khan, 2010). The quality of the semen may decrease as the total volume of the ejaculate increases (Huat, 1976). Older males generally have been reported to have a larger volume of ejaculate than younger males, therefore age should be considered with respect to this parameter (Nuti, 2012).

Concentration, which is expressed as the number of sperm cells per ml is often recorded for each ejaculate as it allows the semen to be split into maximum number of doses of semen required for breeding. In bulls, semen concentration averages about 1.2 billion cells per ml for temperate breeds (Bearden *et al*., 2004) and about 520 million cells per ml for tropical breeds (Osinowo, 1979). In turkeys, spermatozoon concentration range between 6 to 12 billion cells per ml while the concentration in chickens is about one – half that of turkeys (Cynthia and Khan, 2010).

There are different methods used to determine the concentration of spermatozoa in a semen sample. The use of a haemocytometer is the simplest method by which sperm cells are directly counted. Other methods of determining spermatozoa concentration are indirect and include packed cell volume (PCV) or spermatocrit, spectrophotometry and turbidity (Bakst and Long, 2010). These indirect methods required a conversion factor that converts the actual instrument reading to sperm number or concentration in the sample. The conversion factor is usually derived by establishing a regression equation and standard curve which provides a mathematical relationship between the reading provided by the instrument or technique and the actual number of spermatozoa (Bakst and Long, 2010). The concentration of the ejaculate is a function of several parameters

which include the degree of sexual preparation of the male, age, time of the year the collection is made, frequency of semen collection, nutritional status, the general health and production capacity of the male (Nuti, 2012). A concentration dose of 150 to 300 million viable sperm is required for a satisfactory fertilization in turkeys (Cynthia and Khan, 2010).

Motility is expressed as a percentage of cells that are moving under their own power through locomotion of the tail. The speed of a spermatozoa is known as the rate of motility or forward progressive and is often judged on a scale of zero to five as follows: 0 = very poor (0-20%), 1 = poor (20-40%), 2 = fair (40-50%), 3 = good (50-80%), 4 = very good (80-90%), 5 = excellent (90-100%) (Prien, 1991). Percentage motility is estimated at x 10 magnification using a microscope. Cold slide will reduce motility on the other hand, hot slides will kill the spermatozoa. Proper handling techniques specify that the collection tube should be at 37^0C at the time of collection. Motility is best estimated immediately after collection. A stage warmer can be used to maintain the slides at 37^0C while the semen is being examined (Nuti, 2012).

The pH of the semen is measured immediately after collection. It is done using the pH meter. The pH of normal semen should range between 6.5-7.4 (Nuti, 2012). The pH of the semen may vary at different times of the year (Al-Ghalban *et al*., 2004). Anything that alters the pH of the semen above or below the normal range will cause sperm mortality. A low pH may not always be indicative of a poor quality semen. This is because highly active spermatozoa samples produce lactic acid as a metabolic waste product. However, the sperm will be killed if the acidity is not neutralized. Common semen diluents are buffered to balance the pH (Adeyemo *et al*., 2007).

Sperm morphological examination is an important breeding soundness examination (Rouge, 2004). Spermatozoa from different species vary in size and shape, bull and buck spermatozoa have a paddle-shaped head, whereas cocks and turkey toms have spindle- shaped sperm which is almost difficult to distinguish from the mid -piece (Rouge, 2004). It is normal to see some abnormal sperm in an ejaculate but it becomes a thing of concern when the fraction of abnormal spermatozoa becomes excessive (Rouge, 2004). Various staining techniques have been devised for examining sperm morphology. An eosine-negrosin staining is the most commonly used technique because it is effective, simple and allows spermatozoa to be easily visualized. It is also referred to as "live-dead" stain because it allows for the assessment of the membrane integrity at the same time as the morphology (Rouge, 2004). More recently, examination of the morphology of a portion of the head, the acrosome has become popular and it requires the use of highly sophisticated microscope fitted with either a phase – contrast or differential; interference optics. Even though researchers find this measurement helpful it is not routinely performed because of the time and expense in making this measurement (Nuti, 2012).

2.12 Semen Dilution

Semen diluent or extender is a solution designed to keep spermatozoa alive after collection and to dilute the initial concentration of spermatozoa to a desired level for AI (Christensen, 1995). Semen extenders have several functions: optimizing osmotic pressure and pH, providing an energy source to prevent undesirable use of intracellular sperm phospholipids, preventing bacterial contamination during preservation by including antibiotics and increasing the number of animals that can be inseminated per unit volume of semen (Mahony *et al*., 1990).

One of the greatest short coming of AI in the early 1900 was that semen had to be used on the same day of collection. This led Philips and Lardy to discover a buffered nutrient medium which increased the longevity of semen for up to three or four days (Philips and Lardy, 1940). Semen extenders can be placed into three major classes depending upon the species of animals (Bearden *et al.*, 2004). These classes are egg-yolk, milk and serum albumin. Egg-yolk citrate and serum album have been used with success in human and canine semen preservation (Hafez, 1987a: Bearden *et al*., 2004). However, bovine and ovine semen gave better results when only egg-yolk based extenders were used. Serum albumin extenders are used primarily for porcine and exotic animals (Bearden *et al*., 2004).

The choice of a semen diluent is crucial to the viability of the spermatozoa during storage. The basic requirement of a semen diluent is to provide a suitable environment for the survival of a spermatozoa with the least damage to their fertilizing ability (Pano, 1991). The qualities of good semen diluents are: electrolyte balance, stability after prolonged storage, supply of energy to support the metabolic activities of the spermatozoa during storage, good buffering capacity, bacteria control and non toxicity (Salisbury and Van Demark, 1961).

2.13 Dilution of Poultry Semen

The most common procedure for short-term storage of poultry semen requires suspending spermatozoa in an extender to retain their viability *in vitro* (Suidzinska and Lukaszewicz, 2008). Results comparing diluted and un-diluted stored semen showed that applying extenders is necessary to maintain the quality of spermatozoa (Bilgili *et al*., 1987; Lukaszewicz, 1988). An appropriate semen extender for poultry has to

provide an energy source, maintain pH and osmolarity levels identical to those of the seminal plasma (Siudzinska and Lukaszewicz, 2008). Researches aimed at developing the optimal extender for avian semen storage are still in progress since the biochemical composition of seminal plasma was first described (Lake, 1996; Cherms, 1967). There is no standard diluent for the preservation of poultry semen because they are numerous diluents, both published reciepes and commercially available products (Donoghue and Wishart, 2000). Several comparative studies have been carried out on different diluents in the preservation of poultry semen (Howrath Jr, 1983; Bakst, 1990; Bootwala and Miles, 1992).

The physiological differences and the metabolic requirements of spermatozoa from different avian species is important in the development of diluents and storage systems for semen (Donoghue and Wishart, 2000). Chicken spermatozoa are metabolically competent in aerobic and anaerobic environments in vitro, whereas, turkey spermatozoa require high oxygen levels to survive (Sexton, 1974; Wishart, 1981).

Unlike bull, ram and boar spermatozoa, the avian sperm head are cylindrical and not much wider in diameter than the tail with less cytoplasmic volume. It therefore has less ability to move cryprotectants inside the sperm head. This has been interpreted as one of the possible reasons avian spermatozoa do not survive cryopreservation well (Donoghue and Wishart, 2000). The pH of a diluent should be taken into consideration when selecting an extender for the preservation of poultry semen. This is because the pH can affect the metabolic rate and motility of the stored spermatozoa. Buffering agent constituting a mixture of an acid and its conjugate base are formulated into a diluent to limit changes in pH (Donoghue and Wishart, 2000). Chicken and turkey spermatozoa can tolerate a pH range of 6.0-8.0 (Bogdonoff and Schaffner, 1954; Van Wambeke,

1967). A low diluents pH reduces spermatozoa motility, increases lactic acid production and increases oxygen uptake, whereas a high pH increases metabolic rates *in vitro* (Bogdonoff and Schaffner, 1954).

2.13.1 Egg yolk citrate extender

Egg yolk citrate (EYC) diluents was first describe by Salisbury *et al.* (1941). It was described to offer a clear field which allows individual spermatozoa studies and has no depressing effect on the metabolic activities of spermatozoa. Sodium citrate was reported as a good semen extender which performed excellently in maintaining the validity of stored fish semen (Adeyemo *et al*., 2007). The lipoprotein and lecithin from fresh egg yolk and the milk protein casein from milk have been established as agents preventing spermatozoa from cold shock during preservation (Pelicer- Rubio *et al*., 1998).

Egg yolk citrate diluents is prepared by dissolving 2.9g of Sodium citrate salt in 100 ml of distilled water. 80 ml of the solution is measured into a cylinder. Egg yolk should be separated from the egg white. The yolk is dried on a filter paper, then punctured and allowed to drain off into a graduated cylinder containing the buffer to make up 100 ml. penicillin 100,000 units and streptomycin 20,000 mg/ml are added to the solution (Shannon, 1964). The solution is shaken vigorously to allow adequate mixing (Udeh and Oghenesode, 2011). Igboeli (1970) developed a tris (hydroxymethlaminoethane) based diluents for liquid storage of boar semen. Tris based diluents have been reported to perform better than egg-yolk cititrate diluents in preserving ram spermatozoa after 96 hours of storage (Petruzzi *et al.,* 1976). The best result was reported by Adelhakeem *et al*. (1978) that tris based diluents containing 40% egg-yolk and 70% glycerol mentained

spermatozoa motility over 72 hours period better than the best of glucose based diliuents.

Egg yolk citrate diluent is a combination of egg yolk and a buffer, initially phosphate but later replaced by sodium citrate (Miskeje *et al*., 2003). The egg yolk contains nutritional substance that nourishes the spermatozoa and also forms a protective colloid around the spermatozoa from the harmful effect of the external factors (Miskeje *et al*., 2003).

The most important advantage of EYC is the ability to increase the resistance of spermatozoa to thermal shock. This feature is performed by lecithin and the lipid-protein complex contained in the raw egg. The best results was obtained when 20 to 25% of egg yolk was used and it preserved the motility and fertilizing ability of stored semen for 48 hours (Gamcik *et al*., 1976). Numerous special buffered salts have been used as extenders to preserve avian semen (Bootwalla and Miles, 1992). These extenders have different composition, sometimes with special additives such as skimmed milk, egg albumins, gentamycin sulphate, caproic acid or other antibiotics added (Harris *et al*., 1963; Van Wembeke, 1967; Lake and Ravie, 1981 and Sexton *et al*., 1978). Other extenders developed by individual investigators and used for the preservation of avian semen include Lakes extender developed by Lake (1960), EK extender, developed by Lukaszewicz (2002), and extender described by Tselutin *et al.* (1995). Egg yolk citrate extender and the EK extender were reported to give good results in the preservation of turkey semen (Siudzinska and Lukaszewicz, 2008; Miskeje *et al*., 2003).

2.14 Oxidative Stress and Spermatozoa

The term “oxidative stress” is generally used when the oxidants in a system out numbers the antioxidants (du Plessis *et al.,* 2008). It is a state of imbalance between production of reactive oxygen species (ROS) and the ability of a biological system to readily detoxify the reactive intermediates or easily repair the resulting damage (Agarwal *et al.,* 2003). Reactive oxygen species are highly reactive oxidizing agents belonging to the class of free radicals (Aitken and Fisher, 1994) and are produced in excess during oxidative stress (Valko *et al*., 2005). Reactive oxygen species production by spermatozoa is a normal physiological process as ROS has been reported to play important role in physiological processes such as spermatozoa capacitation, acrosome reaction, mainntenance of fertilizing ability stabilization of the mitochondria capsule in the mid-piece of bovine spermatozoa (Agarwal *et al*., 2008; Desai *et al*., 2009; Goncalves *et al*., 2010). However, an inbalance between ROS production and scavenging activity is detrimental to spermatozoa and associated with male infertility (Sharma and Agarwal, 1996). Defective spermatozoa have been reported to produce ROS at an uncontrolled rate with detrimental effects on sperm functions (Baumber *et al.,* 2000). Oxidative stress was reported to be one of the most important factors contributing to poor semen quality (Bucak *et al*., 2010). The succeptibility of spermatozoa to oxidative damage has been attributed to high concentration of sperm per unit volume of seminal fluid and the ability of spermatozoa to generate ROS (Jones and Mann, 1977; Aitken *et al.,* 1989). In addition, spermatozoa are susceptible to ROS induced damage as a result of the polyunsaturated fatty acid (PUFA) rich content of their plasma membrane (Verneet, *et al.,* 2004), and a low concentration of scavenging enzymes in the cytoplasm (de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996). Zini and Sigman, (2009) concluded that the intracellular antioxidants and

enzymes are unable to protect the spermatozoa plasma membrane surrounding the acrosome and the tail, forcing them to supplement their limited intrinsic antioxidant defenses by depending on the protection from the seminal plasma.

2.15 Lipid Peroxidation During *In Vitro* Semen Storage

Lipids are basic component of semen and they play vital role as an energy reserve for spermatozoa metabolism and are involved in the events and functions that lead to fertilization (Tabatabaei *et al*., 2011). Lipid composition of avian semen is unique in its content of long chain polyunsaturated fatty acids (PUFAS) which are essential components of all cell membranes and also give rise to many bioactive molecules (Sardesai, 1992).

Avian spermatozoa are highly susceptible to peroxidative damage because of their high content of PUFAS (Surai *et al*., 1997; 1998a; Zaniboni *et al*., 2006) as well as the ability of spermatozoa to generate ROS (Aitken, *et al.*, 1995; Storey, 1997). Lipid peroxidation is a chain reaction resulting in the formation of lipid peroxides and ultimately the formation of cytotoxic aldehydes (Aitken *et al.*, 1995). Oxidative reaction in biomembranes leads to amplification of ROS, change in membrane fluidity, loss of compartmentalization and plasma membrane integrity, disturbances of ion gradient, impairment of lipid-protein interactions and modification of DNA and proteins (Halliwell and Chiroco, 1993). The damages results from the actions of free radicals and ROS (Zaniboni *et al*., 2006). Oxidative stress is known to play an important role in the etiology of defective sperm functions through the induction of peroxidative damage to the plasma membrane, with a consequent decrease in sperm motility and viability (Griveau *et al*., 1995; Purdy *et al*., 2004; Boonsorn *et al*., 2010).

Oxidative stress results in a fall in intracellular ATP levels which decrease sperm motility and also initiates lipid peroxidation of the PUFA rich sperm plasma membrane. This leads to increased permeability, enzyme inactivation and production of spermicidal end product (Bhakat *et al*., 2011). The production of ROS cannot be completely prevented but incorporating antioxidants into the semen diluent can help minimize it and improve semen motility and viability (Bhakat *et al*., 2011).

In normal physiological function, there is a balanced generation of ROS and antioxidant enzymes (Kovalski *et al.*, 1992). Reactive oxygen species have been reported to play a physiological role in signaling events controlling sperm capacitation and induction of the acrosome reaction in many species (De Lamirande *et al*., 1995). However, over production of ROS and decreased defence activity result in low sperm motility and viability, DNA fragmentation and protein denaturation (Aitken *et al*., 1994b; Kankofer *et al*., 2005). Antioxidants act by reducing the production of deleterious residues from oxidative physiological metabolism. Semen contains a variety of antioxidants acting as free radical scavengers to protect spermatozoa against ROS (Agarwal and Prabakaran, 2005). Semen handling, processing and preservation decreases the antioxidant defence capacity of semen, therefore the addition of antioxidants in the diluents excerts a protective effect against lipid peroxidation thereby preserving the motility and viability of preserved semen (Jiang *et al*., 2007; Kadirvel *et al*., 2009).

Reactive oxygen species are produced continuously by spermatozoa during *in vitro* storage and are neutralized by antioxidants present in the semen (Sharma *et al*., 1999). However, when the ROS production exceeds the capacity of the antioxidants, a state of oxidants stress is created (Ahmad and Baig, 2011).

2.16 Antioxidative Defense of Avian Spermatozoa

The oxidants system present in avian seminal plasma has been reported by series of recent studies. These studies revealed the presence of a complex system involving vitamin E, vitamin C and glutathione (GSH) in the seminal plasma (Surai *et al*., 1997; 1998c; 2000). Vitamin E and GSH concentration in spermatozoa was reported to be nine times higher than those observed in seminal plasma but different from vitamin C which is equally distributed between the seminal plasma and the spermatozoa (Surai *et al*., 1998c). Based on the findings, it has been suggested that vitamin E has a minor role with regards to its antioxidants potential in seminal plasma while vitamin C might have a major role as a water soluble vitamin (Surai *et al*., 1998c). The antioxidants work in close association with series of antioxidants enzymes such as glutathione peroxidase (GSH-PX) and Superoxide Dismutase (SOD) (Mannella and Jones, 1980; Surai *et al*., 1998a; b; 2000).

A 3-tier level of defense was proposed in which SOD associated with GSH-PX and metal binding proteins represent the first level responsible for the prevention of free radical formation (Surai, 1999; 2000; 2002a). A secondary level comprising natural antioxidants (vitamin A, vitamin C, vitamin E, uric acid, GSH, carotnoid e.t.c) associated with GSH-PX prevent and restricts the formation and propagation of peroxides. The third level is based on the association of several enzymes responsible for the repair and removal of damaged molecules in cell membranes (Breque *et al*., 2003).

It is note worthy that despite these highly organized lines of defense against lipid peroxidation by the avian spermatozoa, the antioxidant systems are unable to fully

prevent the negative effects of lipid peroxidation during prolonged *in vitro* storage (Breque *et al*., 2003).

Results on the antioxidant systems in the semen of mammals have been documented (Jones and Mann, 1977; Dawara *et al*, 1983; Kobayashi *et al*, 1991; Castellini *et al.*, 2000). Fujihara and Koga (1984) reported that the seminal plasma in chickens inhibit lipid peroxidation of sperm membrane during *in vitro* storage at 0ºC. This finding was supported with similar results in turkeys (Cecil and Baskt, 1993). The free radical-trapping activity of chicken seminal plasma was reported to be more potent than the antioxidant activity of chicken blood plasma (Surai *et al.*, 1998c), but not as potent as that observed in the seminal plasma of turkeys and ducks (Surai *et al.*, 1998d; 2000).

It has been established that the total antioxidant activity of seminal plasma is higher in turkeys than in other poultry species. In contrast, the antioxidant activities developed by the enzymatic system present in semen of turkeys are less potent than those found in chickens, ducks and ganders (Surai *et al.*, 1998c). These findings suggest a species–specific regulation of semen susceptibility to *in vivo* lipid peroxidation.

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2.17 Measuerement of Lipid Peroxidation

Lipid peroxidation is measured by determining the malondialdehyde (MDA) production. Malondialdehyde is an end-product of lipid peroxidation and its measurement represents the level of lipid peroxidation (Ahmad and Baig, 2011). High lipid peroxidation represented by MDA levels is suggested to be the cause of the deteriorative changes in spermatozoa and diminished fertility (Geva *et al*., 1996). In turkey semen, MDA production was found to be 10 fold higher in toms after 26 weeks

of semen production compared to males after 2 weeks of semen production (Donoghue and Donoghue, 1997).

Malondialdehyde is measured by the thiobutyric acid (TBARS) method or the modified calometric assay (Rao *et al*., 1989; Long and Kramert, 2003; Ahmad and Baig, 2011). Roa *et al*., (1989) described the thiobutyric acid (TBARS) method in details stating its specificity and sensitivity over other methods of MDA measurement. To measure the MDA using the TBARS method, each diluted sperm suspension is mixed with 2 ml of TBA reagent (15% trichloro acetic acid + 0.25 NHCL) in a sterile caped tube and incubated in a water bath at 100^0C for 15 minutes. After cooling, the suspension is centrifuged at 1000 g for 10 minutes and the optical density of the supernatant measured at 535 nm using a spectrophotometer. The MDA concentration is measured in µl/ ml (Rao *et al*., 1989; Ahmad and Baig, 2011).

2.18 Antioxidant Preservatives

2.18.1 Vitamin C

Vitamin C or ascorbic acid is a six carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not by humans, non-human primates and guinea pigs (Sebastian *et al.,* 2003). The inability to synthesize vitamin C by these species have been attributed to the absence of the enzyme gulonolactone oxidase which is important for the synthesis of 2-keto-1-gulonolactone, an immediate precussor of vitamin C (Nishikimi and Yagi, 1996). It functions *in vivo* as a co-factor for at least eight enzymatic reaction including several collagen synthesis reaction that when dysfunctional, causes symptoms of scurvy and can also act as an antioxidant by reacting with free radicals (Carr and Frei, 2002; Micheal *et al*., 2008).

Majority of animals and plants are able to synthesize vitamin C through a sequence of enzyme- driven steps which convert monosaccharides to vitamin C. In plants, this is accomplished through the conversion of mannose or galactose to ascorbic acid (Wheeler *et al*., 1998). In some animals, glucose is extracted from glycogen for the synthesis of ascorbic acid. Hence ascorbic acid synthesis is a glycogenolysis-dependent process (Banhegyi and Mandl, 2001). All living animals either synthesize ascorbic acid, eat it or may die from scurvy due to lack of it. Reptiles and older order of birds produce ascorbic acid in their kidneys. Recent orders of birds produce ascorbic acid in their liver through the action of the enzyme L-gulonolactone oxidase which converts glucose to ascorbic acid (McClauskey, 1985). Ascorbic acid is an effective antioxidant acting to prevent or lessen oxidative stress (Padayatty *et al*., 2003). Ascorbic acid, even in small amounts can protect vital molecules in the body such as protein, lipid, carbohydrates and nucleic acid from possible damages caused by free radicals and ROS that can be generated during normal metabolism as well as through exposure to toxins and pollutants (Tak *et al*., 2000). Ascorbic acid has been reported to ameliorate heat stress and adverse effects of environmental conditions (Minka and Ayo, 2007; 2008).

All known physiological and biological actions of vitamin C are due to its action as an electron donor (Sebastian *et al*., 2003). Ascorbic acid was reported to be the most important antioxidant in the seminal Plasma (Asadpour *et al*., 2002), with a concentration of about 10 times greater than in blood plasma (Chow, 1991; Nikki 1991; Lewis *et al*., 1997).

2.18.2 Mechanism of action

Vitamin C acts as an electron donor and therefore is a reducing agent and can donate a pair of electrons from the double bond between the second and third carbons of the 6 carbon molecule, and in the process prevents other compounds from being oxidized while itself is oxidized in this reaction (Sebastian *et al*., 2003). Ascorbic acid reacts with a reactive free radical through a reduction reaction forming semidehydroascorbic acid or ascorbyl radical. The ascorbyl radical is relatively stable with a half life of 10^{-5} seconds (Sebastian *et al.*, 2003). Ascorbic acid is therefore a good free radical scavenger due to this chemical property (Bieski *et al*., 1975; Buettner and Moseley, 1993). Vitamin C, being a water-soluble vitamin is a powerful anti-oxidant when peroxyl radicals are located in the aqueous phase but the vitamin is a poor scavenger for ROS within the lipid membrane (Burton and Ingold., 1986). However, the demonstration that vitamin C may interact with the alpha-tocopheroxyl radical to regenerate alpha-tocopherol provided evidence that vitamin C also serves indirectly in the protection of membrane components from peroxidative damage (Packer *et al*., 1979; McCay, 1985; Beyer, 1994). *In vitro* experiments conducted to access the effect of ascorbic acid on low density lipid (LDL) oxidation revealed a delay in oxidation by the addition of ascorbic acid (Frei, 1991; Jialal and Grundy, 1992; Frei and Gazaino, 1993).

The addition of vitamin C to an extender was shown to impact optimal sperm performance by reducing spermatozoa damage through its continuous radical-scavenging action (Asadpour *et al*., 2002). Beconi *et al*. (1993) reported that the addition of 5 mg of vitamin C into semen diluent exerted an antioxidant effect during freezing and thawing of bovine semen. Al-Daraji (2002) reported significant improvement in spermatozoa integrity, viability and motility for 24 hours at 4^0C when

the diluents was supplemented with vitamin C. Beconi *et al.* (1993) observed that at low concentration, vitamin C exibited antioxidant properties. However, Girotti *et al.* (1985) earlier reported that higher concentrations of ascorbic acid have the ability to form free radicals and initiate lipid peroxidation *in vitro.* This finding shows that vitamin C could act as a prooxidant and an antioxidant depending on its concentration. Al-Daraji (2002) reported an increase in mass motility, individuals motility and viability result when the diluents of poultry semen was supplemented with 4 mg and 8 mg of vitamin C.

2.19 Vitamin E

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienes which are fat soluble vitamins with antioxidant properties (Packer *et al*., 2001; Herrera and Barbas, 2001). One of the earliest observations of the physiological effect of vitamin E relates to reproduction. The association of vitamin E with impaired male function has been long established and hence it is traditionally called an "anti-sterility" vitamin (Tabatabaei *et al*., 2011). Vitamin E deficient models showed abortion and fetal resorbtion in female animals while atrophied testis was reported in male animals (Nelson, 1980). Sharma and Agarwal (1996) reported vitamin E to be the main chain breaking antioxidant in membranes because it directly scavenges free radical such as superoxide anions, hydrogen peroxide and hydroxyl radical. Vitamin E was also reported to ameliorate oxidative stress in spermatozoa helping to maintain optimum fertilizing ability (Surai and Ivanov, 1992). Vitamin E is believed to be the primary component of the antioxidant system of spermatozoa and is also one of the major membrane protectants against ROS and lipoperoxidative attack (Yousef *et al*., 2003). It also appears to be the first line of defense against the peroxidation of PUFAS contained in the cellular and sub-cellular membrane phospholipids because of its lipid solubility (Horton *et al*., 2002). Alpha tocophenol was reported to be the most important lipid

soluble antioxidant that protects cell membrane from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber, 1999; Herrera and Barbas, 2001).

2.19.1 Mechanism of action of vitamin E

Alpha tocopherol has been reported to scavenge and remove free radicals and their intermediates, thereby terminating the oxidation reaction (Brigelius-Flohe and Traber, 1999). Alpha tocopherol reacts with free radicals such as superoxides, hydroxyl ions and nitric oxide, which all possess an unpaired electron. The phenolic hydroxyl group of alpha tocopherol reacts with an organic peroxyl radical to form the corresponding organic hydroperoxide and the tocopheroxyl radical (Traber and Sies, 1996). It reacts by binding to these free radicals and neutralizing its unpaired electron to form an intermediate product which is relatively stable and less reactive (Halliwell and Gutteridge, 1999). Alpha tocopherol maintains the integrity of cell membranes by preventing the chain reaction of lipid oxidation. When alpha tocopherol neutralizes a free radical, it is altered in such a way that its antioxidant capacity is lost. However, other antioxidants, such as vitamin C are capable of regenerating the antioxidant capacity of alpha tocopherol (Traber , 2006; Bruno *et al.*, 2006).

Antioxidant vitamin E is present in turkey semen with the highest concentration localized within sperm cells than in seminal plasma (Surai, 1981). Vitamin E has been reported to be a natural stabilizer of poultry spermatozoa plasma membrane (Surai and Ivanov, 1992), and has been demonstrated to improve turkey sperm motility and viability during storage at 5^0C when incorporated into the extender (Donoghue and Donoghue, 1997). Al-Daraji, (2002) reported a good result upon supplementing rooster

semen extender with 4 mg and 8 mg/ml of vitamin E as compared to 0, 12 and 16 mg/ml of vitamin E.

2.20 Antioxidant Network

Antioxidants do not function in isolation, rather they take part in an inter-linking set of redox antioxidant cycle termed "antioxidant network" (Constantinescu *et al*., 1993). It is hypothesized that vitamin E is reduced from its free radical form which arises after binding and neutralizing lipid radicals to its native state (Packer *et al*., 2001). This reaction occur through the interaction of water and lipid soluble substances by both non enzymatic and enzymatic mechanism that regenerate vitamin E from its tecotrienoxyl or tocopheroxyl radical back to tocotrienol and tocopherol respectively (Parker *et al*., 2001). Ascorbic acid and vitamin E have been labeled "partners in defense" in a review by Chan (1993). He went further to explain the non- enzymatic regeneration of vitamin E by vitamin C and the enzymatic regeneration of the hydrophobic phase of vitamin E by glutathione. Ascorbic acid scavenges free radicals generated during lipid peroxidation and reduces tocopheroxyl radical into tocopherol (Beyer, 1994; Jacob, 1995). Glutathione further reduces the dehydroascorbic acid produced in these reactions (Thurman *et al*., 1999).

Majority of studies carried out on vitamin C and E, noted a synergistic action of the two vitamins (Hiramatsu *et al*., 1990; Mukai *et al*., 1991; Glascott *et al*., 1995). This is evidenced by a worsened clinical feature of the deficiency of vitamin E with a concurrent state of deficiency of vitamin C (Hill *et al.,* 2001). Asadpour *et al*. (2002) concluded that vitamin C and E are very efficient antioxidants and their addition to an extender can reduce the oxidative stress provoked by semen handling and preservation.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was carried out in the Department of Theriogenology and Production, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, situated in the Northern Guinea Savannah, between latitude 11^0 1573' N and longitude 7^0 64989' E at an elevation of 646 m above sea level. The mean annual rainfall in this area is 1,100 mm lasting from May to October. Mean daily temperature during season is 25 ^{0}C with a mean relative humidity of 72%. The dry season last from November to April, with mean daily temperature ranges of 14 – 36^{0}C and relative humidity of 20 – 30% (Anonymous, 2014).

3.2 Experimental Turkey Toms

Twenty apparently healthy indigenous turkey toms aged between 37-38 weeks were used for this study. The turkey toms were sourced from local markets within Zaria. The toms were weighed, screened and treated for helminthes and blood parasites prior to the onset of the study. The toms were randomly allocated into four treatment groups T_0(Control), T_1 (vitamin C), T_2 (vitamin E) and T_3 (vitamin C+E) of five toms each.

3.3 Housing and Management of the Turkeys

Turkey toms were housed individually in cages and allowed to acclimatize for a period of one month during which they were trained for semen collection. They were fed with compounded turkey grower diet containing; maize, 45%; soya bean cake, 43%; fish meal, 4%; palm oil, 3%; Bone meal, 3%; salt, 0.5% and premix, 0.5%. The feed was

analysed at the Animal Nutrition Laboratory Department of Animal Science, A.B.U, Zaria. Water was provided *ad-libitum.*

3.4 Initial Semen Collection and Evaluation

Semen was collected from the toms by abdominal massage technique as described by Burrows and Quinn (1937) with modification. Each tom was placed in between the arm and the body of the restrainer with its legs gently restrained as shown in Plate 3.I. The handler firmly massages the tom's abdomen with one hand while concurrently stroking firmly the back and tail feathers with the other hand. After a few seconds, the phallus should enlarge slightly (Phallic tumescence) and the handler will move to straddle the cloaca with the thumb and forefinger of the hand situated just above the cloaca squeezing upward and inward as shown in Plate 3. II. This action, referred to as a "cloacal stroke" will result in a partial ejaculation. It was repeated a second time to complete the ejaculation process. The semen was collected into a calibrated semen vials and evaluated for volume (Plate 3.III), colour, pH, concentration, motility (gross and individual), live-dead ratio and morphology.

3.5 Semen Evaluation

Collected semen samples were evaluated as described by Baskt and Long (2010) for:

- Volume**:** The volume was measured immediately after collection using a calibrated tube (Plate 3.III).
- Gross motility**:** Microscopic examination for wave pattern was carried out by placing a drop of raw undiluted semen on a pre-warmed glass slide then cover-slipped and viewed for motility using a microscope at x4 and x10 objective magnification (Plate 3.VIII).

- Sperm concentration**:** The semen concentration was determined using a haemocytometer as described by Azawi and Ismaeel (2012). Micropipette was used to aspirate the semen to 0.5 mark and physiological saline aspirated to make up to 1.0 mark, hence diluting the semen. The pipette was wiped to remove any adhering semen. The first three drops was discarded, 2 drops was placed on either margin of the haemocytometer, cover-slipped and left for 5 minutes. It was then examined using a microscope at x 40 magnification and then sperm cells counted in five squares of one chamber of the haemocytometer. The semen concentration was calculated as follows:

  Concentration (sperm cells/ml) = Average count x dilution factor $x10^4$
- Live spermatozoa**:** was determined as described by Esteso *et al.* (2006). A drop of semen sample was placed on the edge of a clean grease free glass slide and three drops of eosine-negrosine stain was mixed with the semen. A smear of the mixture was made on a clean slide. Live (viable) spermatozoa have intact cell membranes which are capable of of excluding eosin dye and remain colorless. Dead spermatozoa (non-viable) on the other hand possess permeable membrane which allows the spermatozoa to pick up the eosin dye and stain pinkish. Two field was counted using a microscope at x 40 magnification and expressed as percentage live.
- Sperm abnormalities: Sperm abnormalities were determined by making a thin smear from the mixture of semen sample and eosine-negrosine on clean grease free glass slide. One hundred sperm cells were counted per slide using light microscope at x 40 magnification (Esteso *et al*., 2006).



Plate I. Restrain for semen collection



Plate II. Semen collection (Arrow showing semen collection from cloaca)



Plate III: Measurement of semen volume (arrow pointed at semen in graduated test tube)

3.6 Malondialdehyde Assay

Malondialdehyde was measured by the Thio Butyric Acid Reactive Substances (TBARS) method as described by Rao *et al*. (1989). A detection kit was purchased from Radox (Radox laboratory Ltd, United Kingdom). 0.1 µl of semen was mixed with 0.1 µl of TBA reagent (15% trichloro acetic acid + 0.25NHCL). A coloured product was formed. The optical density was measured at 450 nm using a spectrophotometer (Rayto, Japan) and the MDA concentration was measured in µl /ml.

3.7 Diluted Semen Evaluation

3.7.1 Diluent preparation

Egg yolk citrate diluent was prepared at ambient temperature as described by Salisbury (1941). by measuring 2.9 grams of sodium citrate into 100 millilitres of sterile water i.e., 2.9 % w/v with the pH adjusted between 6.9 – 7.6. Fresh chicken egg was obtained and washed in warm water with mild detergent, properly rinsed and dried with a clean dry towel. To make 100 ml of the extender, 80 millilitre of sodium citrate was measured into a graduated cylinder. The egg was broken midway and the albumin discarded. The yolk was placed on a filter paper (Plate 3.IV) to remove as much as possible the albumin before being punctured. The yolk membrane was punctured with a sterile glass rod and the yolk was expelled into the graduated cylinder containing the extender to the 100 millilitre mark. The yolk membrane was then discarded. The measuring cylinder was inverted severally for thorough mixing. Antibiotics (penicillin G; 1000 iu/ml and streptomycin (1g/ml) was added to the mixture. The contents were mixed properly (Plate 3.V and 3.VI).

3.8 Semen Extension and Evaluation

Semen was collected from individual turkey toms in the various groups and subjected to gross evaluation prior to pooling. Egg yolk citrate extender was prepared at room temperature, and divided into 4 parts which were supplemented with the antioxidant vitamins C (4 mg/ml), E (8 mg/ml), C+E (4 mg + 8 mg/ml), respectively and control corresponding to groups T_1, T_2, T_3 and T_0, respectively. No antioxidant was added to the extender in the control group (T_0). Semen collected from individual tom within each group was pooled, mixed and diluted with EYC extender at a dilution rate of 1:100. The diluents-semen mixture was swerved gently to facilitate proper mixing. The samples were packed into labelled bijou bottles and placed in a padded flask and then transferred into a refrigerator at 4^0C for storage.

Malondialdehyde assay, sperm motility, morphological abnormalities and viability (% live) were measured after dilution at 0, 3, 6, 9, 12, 24 and 48 hours of storage. The procedure was repeated thrice with mean values obtained.

3.9 Data Analyses

Data collected from this study were expressed as means ± standard errors of means (SEM). One way Analysis of Variance (ANOVA) was used for the analysis of the data, followed by Turkey's multiple comparison tests. Values of $p < 0.05$ were considered significant. All statistical analysis was done using GraphPad Prism Software version 5.0.

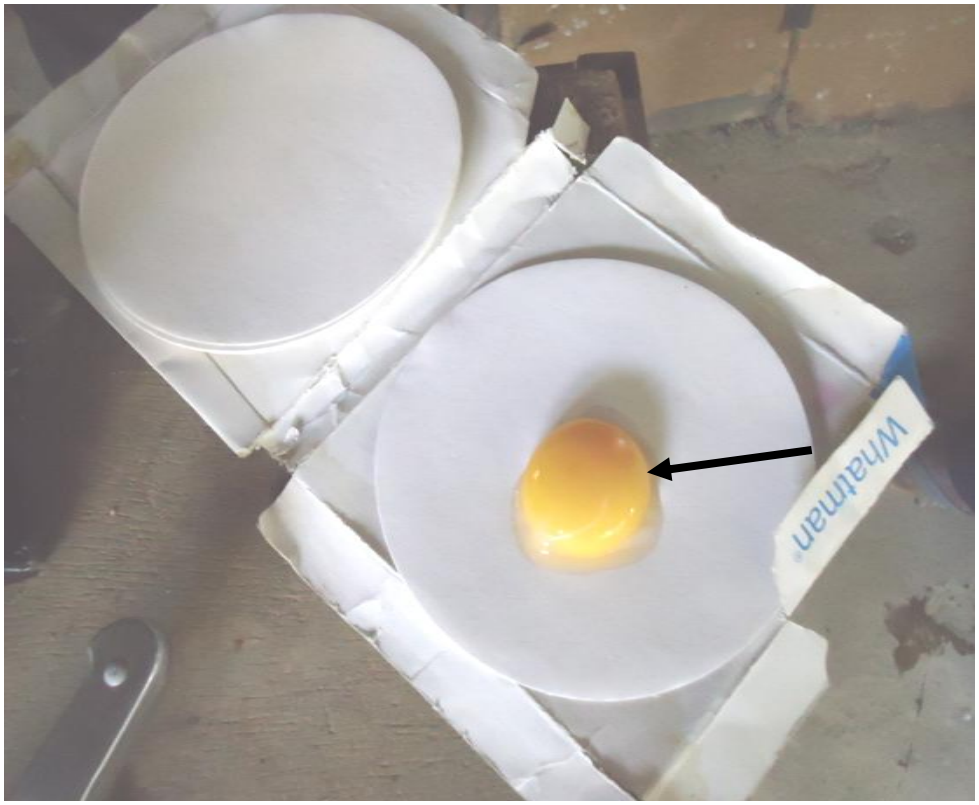


Plate IV. Egg yolk placed on a filter paper to remove albumen



Plate V: Diluent preparation.



Plate VI: Completion of diluent preparation



Plate VII: Reading pH of diluents (arrow)



Plate VIII: Microscopic examination of collected semen



Plate IX: Diluent in bijjou bottles with some in graduated tubes (arrows (a) =diluted semen in bijjou bottles (b)= diluted semen in graduated test-tube)

CHAPTER FOUR

RESULTS

4.1 Baseline Spermiogram of Nigerian Indigenous Turkey Toms

The baseline spermiogram of indigenous turkey toms obtained from the study is presented in Table 4.1. The mean ± SEM of semen volume ranges was 0.17± 0.01-0.24±0.02 ml. The colour was averagely creamy to milky 2.43 ± 0.10. The mean spermatozoa motility, pH, concentration, per cent live, percent deformed and MDA were 91.75 ± 0.97%, 6.94 ± 0.10, 6.80 ± 0.09 $x10^9$ sperm/ml, 91.75 ± 0.90%, 8.15 ± 0.59% and 0.74 ± 0.04 µl/ml respectively. Plate 4.I, 4.II and 4.III shows micrograph slides of live spermatozoa, dead spermatozoa and spermatozoa abnormalities, respectively.

Table 4.1. Baseline spermiogram of indigenous turkey toms

Semen traits	Treatment groups			
	T0	T1	T2	T3
	(n=5)	(n=5)	(n=5)	(n=5)
Volume (ml)	0.24±0.02	0.17±0.01	0.18±0.01	0.18±0.02
Colour	2.74±0.10	2.15±0.10	2.50±0.11	2.35±0.10
Motility (%)	89.75±1.05	89.50±1.02	91.00±1.00	91.75±0.97
Conc (x 10^9/ml)	6.90±0.07	6.97±0.02	6.85±0.08	6.80±0.0
pH	6.71±0.01	6.83±0.10	6.92±0.02	6.94±0.10
% Live	86.30±1.61	92.65±1.06	91.47±0.80	91.75±0.90
% Deformed	7.15±0.63	7.05±0.66	8.75±0.74	8.15±0.59
MDA (µl/ml)	0.72±0.03	0.73±0.03	0.72±0.03	0.74±0.03

4.2 Effects of Vitamin C, E and Their Combination on the Motility of Chilled Turkey Spermatozoa

Spermatozoa motility decreased significantly $p < 0.05$ across the groups with increase in storage time regardless of the antioxidants added to the diluents as shown in Table 4.2. At 0 hour post semen dilution, spermatozoa motility were 91.7±2.9%, 81.7±2.9%, 93.3±2.9%, and 85.0±8.7% for T0, T1, T2 and T3 respectively, while at 3 hours post dilution, spermatozoa motility were 75.0±2.9%, 76.7±1.7%, 86.7±3.3%, and 71.7±1.7% for T0, T1, T2 and T3 respectively. At 6 hours post dilution, spermatozoa motility reduced to 68.3±1.7%, 70.0±0.0%, 80.0±2.9% and 65.0±2.9% for T0, T1, T2 and T3 respectively. The motility at 9 hours was 55.0±2.9%, 63.3±2.9%, 76.7±2.9% and 60.0±2.9% for T0, T1, T2 and T3 respectively. At 12 hours, the motility were 38.3±4.4%, 46.7±1.7%, 65.0±2.9% and 50.0±0.0% for T0, T1, T2 and T3 respectively. At 24 hours post dilution, spermatozoa motility reduced to 0.0±0.0%, 0.0±0.0%, 55.0±2.9% and 10.0±5.7% for T0, T1, T2 and T3 respectively. The lowest motility result was obtained at 48 hours post dilution (0.0±0.0%, 0.0±0.0%, 8.3±4.4% and 0.0±0.0% for T0, T1, T2 and T3 respectively.

Table 4.2. Effects of vitamin C, E and their combination on the motility of chilled turkey Spermatozoa

Time (Hr)	T0 n(5)	T1 n(5)	T2 n(5)	T3 n(5)
0	91.7 ± 2.9 [a]	81.7 ± 2.9 [a]	93.3 ± 2.9 [a]	85.0 ± 8.6 [a]
3	75.0 ± 2.9 [a]	76.7 ± 1.7 [a]	86.6 ± 3.3 [b]	71.6 ± 1.7 [a]
6	68.3 ± 1.6 [a]	70.0 ± 0.0 [a]	80.0± 2.9 [b]	65.0 ± 2.9 [a]
9	55.0 ± 2.9 [a]	63.3 ± 2.9 [a]	76.6 ± 2.9 [b]	60.0 ± 2.9 [a]
12	38.3 ± 4.4 [a]	46.6 ±1.7 [a]	65.0 ± 2.9 [c]	50.0 ± 0.0 [a]
24	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	55.0 ± 2.9 [b]	10.0 ± 5.7 [a]
48	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	8.3 ± 4.4 [a]	0.0 ± 0.0 [a]

Mean values with different superscript ([a, b, and c]) are significantly ($p < 0.05$) different

4.3 Effects of Vitamins C, E and Their Combination on the Viability of Chilled Turkey Semen

The viability of diluted chilled turkey semen as represented by per cent live is shown in Table 4.3. The per cent live spermatozoa at 0 hour post dilution were 94.7±0.9%, 93.0±2.5%, 92.3 ±1.4% and 90.3 ± 1.5% for T0, T1, T2 and T3, respectively. At 3 hours post dilution, the percent live spermatozoa were 81.7 ± 1.5%, 84.0 ± 0.6, 89.7 ± 0.9% and 86.3 ± 1.8% for T0, T1, T2 and T3 respectively. At 6 hours post dilution, the percent live results were 61.7 ± 1.8%, 75.0 ± 0.8, 83.0 ±1.5% and 78.0 ± 1.5% for T1, T2, T3 and T4 respectively. At 9 hours post dilution, the per cent live spermatozoa were 55.0 ±2.9%, 65.0 ± 2.6%, 71.0 ± 2.3% and 69.7 ± 4.7% for T1,T2,T3 and T4 respectively. At 12 hours post dilution, the per cent live spermatozoa were 35.3 ± 3.9%, 47.0 ±2.6%, and 55.0 ± 2.6% and 47.0 ±3.8% for T1, T2, T3, and T4 respectively. The percent live spermatozoa at 24 hours post dilution was 10.3 ± 1.2%, 19.0 ± 3.5%, 41.3 ± 1.5% and 25.0 ±4.0%. The lowest per cent live result was obtained at 48 hours post dilution (0.0 ±0.0%, 4.0 ± 0.6%, 8.3 ± 0.9% and 3.7 ± 0.9% for T1, T2, T3 and T4, respectively).

Table 4.3. Effects of vitamins C, E and their combination on the viability of chilled turkey Semen

Time (Hr)	T0 n(5)	T1 n(5)	T2 n(5)	T3 n(5)
0	94.6 ± 0.9 [a]	93.0 ± 2.5 [a]	92.3 ± 1.4 [a]	90.3 ± 1.4 [a]
3	81.6 ± 1.4 [a]	84.0 ± 0.6 [a c]	89.6 ± 0.9 [b c]	86.3 ± 1.7 [a c]
6	61.6 ± 1.7 [a]	75.0 ± 2.0 [bx]	83.0 ± 1.1 [c y]	78.0 ± 1.5 [d x y]
9	55.0 ± 2.9 [a]	65.0 ± 2.6 [a c]	71.0 ± 2.3 [b c]	69.6 ± 4.7 [a c]
12	35.3 ± 3.9 [a]	47.0 ± 2.6 [a c]	55.0 ± 2.6 [b c]	47.3 ± 3.8 [a c]
24	10.3 ± 1.2 [a]	19.0 ± 3.4 [a y]	41.3 ± 1.4 [b]	25.0 ± 4.0 [c y]
48	0.0 ± 0.0 [a]	4.0 ± 0.6 [b x]	8.3 ± 0.9 [c y]	3.6 ± 0.9 [d x y]

Mean values with different superscript ([a,b,c, x and y]) are significantly ($p < 0.05$) different

4.4 Effects of Vitamins C, E and their Combination on Morphological Defects of Chilled Turkey Semen

The morphological defects of chilled turkey spermatozoa is as shown in Table 4.4. At 0 hour post-dilution, there was no significant differences ($p > 0.05$) between the mean morphological sperm defects across the treatment groups and the control. At 3 hour post dilution, the sperm morphological defects of T1, T2 and T3 (13.0±0.6%, 11.3±0.9% and 11.7±0.7%) were not significantly ($p >0.05$) different from the control (14.0±1.1%). At 6 hour post dilution, the mean morphological defects of T2 and T3 (14.3± 0.7% and 16.0 ±0.6%) were significantly ($p <0.05$) lower than T1 and T0 (17.0 ±0.7 and 20.0± 1.2%), respectively. The mean morphological defects of T1 was lower than T0 (control). However, the difference was not significant ($p >0.05$). At 9 hours post dilution, the morphological defects of T1, T2 and T3 (21 ± 0.6%, 16-3 ± 0.7% and 18.0 ± 0.6%) were significantly ($p <0.05$) lower than the control (T0) (25.3± 1.3%). There was also a significant ($p <0.05$) difference in the mean sperm defects between T1 and T2 (21± 0.6% and 16.3± 0.7%). However, there was no significant ($p >0.05$) difference in the mean sperm defects between T1, T3 and T2 , T3. At 12 hours post dilution, the mean sperm defects of T1, T2 and T3 (24.7 ± 1.2%, 20.7 ± 0.9% and 23.0 ± 1.1) were significantly ($p <0.05$) lower than the control (T0). However, there was no significant ($p >0.05$) between the mean sperm defects of T1 and T2 (24.7 ± 1.2% and 20.7±), T1 and T3(24.7± 1.2% and 23.0± 1.1%) and between T2 and T3 (20.7± 0.9% and 23.0 ± 1.1%). At 24 hours post dilution, the mean sperm defects of T1, T2 and T3 (30.3± 0.9%, 24.3± 0.3% and 26.7± 0.3%) were significantly ($p <0.05$) lower than the control (T0) (42.7± 2.3%). There was no significant ($p <0.05$) difference between the mean sperm defects of T1 and T3 (30.3 ± 0.9% and 26.7± 0.3%) and also between T2 and T3 (24.3± 0.3% and 26.7 ± 0.3%). At 48 hour post dilution, the mean sperm defects of T1, T2 and T3 (36.0 ± 0.6%, 29.3 ± 0.7% and 31.3 ± 0.9%) were significantly ($p <0.05$)

lower than the control (T0). There was no significant difference in the mean sperm defects between T1and T3 (36.0 ± 0.6% and 31.3 ± 0.9%) and also between T2 and T3 (29.3±0.7% and 31.3± 0.9%).

Table 4.4. Effect of vitamin C, E and their combination on morphological defects in chilled turkey semen

	Spermatozoa Morphological deformities (%)			
Time (Hr).	T0	T1	T2	T3
0	9.3 ± 0.9 [a]	8.6 ±1.2 [a]	9.0 ± 0.6 [a]	8.6 ± 1.5 [a]
3	14.0 ± 1.1 [a]	13.0 ± 0.6 [a]	11.3 ± 0.9 [a]	11.7 ± 0.7 [a]
6	20.7 ± 1.2 [a]	17.7 ± 0.7 [ax]	14.3 ± 0.7 [bx]	16.0 ± 0.6 [c x]
9	25.3 ± 1.3 [a]	21.0 ± 0.6 [by]	16.3 ± 0.7 [c x]	18.0 ± 0.6 [d xy]
12	29.7 ± 0.3 [a]	24.7 ± 1.2 [b x]	20.7 ± 0.9 [c x]	23.0 ± 1.1 [d x]
24	42.7 ± 2.3 [a]	30.3 ± 0.9 [b x]	24.3 ± 0.3 [c x]	26.7 ± 0.3 [dc x]
48	55.0 ±1.7 [a]	36.0 ± 0.6 [b x y]	29.3 ± 0.7 [c x]	31.3 ± 0.9 [d x]

Mean values with different superscript ([a,b, c, d, x and y]) are significantly ($p < 0.05$) different

4.5 Effect of Vitamin C, E and their combination on Malondialdehyde concentration in chilled turkey semen

Malondialdehyde concentration was measured over a 48 hour period of study as shown in Table 4.5. Regardless of the treatment, MDA concentration increased with storage time. The mean concentration at 0 hour were 2.1 ± 1.8 µl/ml, 1.2 ± 0.3 µl/ml, 0.9 ± 0.1 µl/ml and 0.9 ± 0.1 µl/ml for T0, T1, T2 and T3, respectively. At 3 hours post dilution, the mean concentration increased to 2.2 ± 0.1 µl/ml, 1.3 ± 0.3 µl/ml, 1.1 ± 0.9 µl/ml and 1.1 ± 1.2 µl/ml for T0, T1, T2 and T3 respectively. After 6 hours post dilution, there was a further increase in the mean MDA concentration to 2.2± 0.4 µl/ml, 1.3 ± 0.5 µl/ml, 1.1 ± 0.9 µl/ml and 1.4 ± 0.2 µl/ml for T0, T1, T2 and T3, respectively. At 9 hours post dilution, the mean MDA concentration were 2.6 ± 0.9 µl/ml, 1.7 ± 0.1 µl/ml, 1.4 ± 0.2 µl/ml and 1.8 ± 0.8 µl/ml for T0, T1, T2 and T3, respectively. At 12 hours post dilution, the mean MDA concentration was 2.9 ± 0.0 µl/ml, 2.0 ± 0.1 µl/ml, 1.7 ± 0.9 µl/ml and 2.1 ± 0.2 for T0, T1, T2 and T3, respectively. At 24 hours, there was a further increase in mean MDA concentration across the groups (3.1 ± 0.2 µl, 2.7 ± 0.2 µl, 2.5 ± 0.3 µl and 2.2 ± 0.2 µl for T0, T1, T2 and T3, respectively). 48 hours post dilution, the mean MDA concentration were 3.1 ± 0.2, 2.7 ± 0.3, 2.1 ± 0.1 and 2.2 ± 0.3 for T0, T1, T2 and T3, respectively.

Table 4.5. Effect of vitamin C, E and their combination on MDA concentrations in chilled turkey semen

Malondialdehyde concentration (µl/ml)				
Time (Hr)	T0	T1	T2	T3
0	2.13 ± 0.2 [a]	1.2 ± 0.3 [b x]	0.90 ± 0.1 [c x]	0.93 ± 0.1 [d x]
3	2.2 ± 0.1 [a]	1.3± 0.3 [a x]	1.1 ± 0.0 [b x]	1.0 ± 0.1 [c x]
6	2.2 ± 0.4 [a]	1.3 ± 0.4 [a]	1.1 ± 0.1 [a]	0.9 ± 0.2 [a]
9	2.5 ± 0.0 [a]	1.7 ± 0.1 [b x]	1.4 ± 0.2 [c x]	1.7 ± 0.1 [d x]
12	2.9 ± 0.0 [a]	2.0 ± 0.0 [b x]	1.7 ± 0.1 [c x]	2.1± 0.2 [d x]
24	3.1 ± 0.1 [a]	2.7 ± 0.2 [a]	2.5 ± 0.2 [a]	2.1 ± 0.1 [a]
48	3.0 ± 0.1 [a]	2.7 ± 0.2 [a]	2.1 ± 0.0 [a]	2.2 ± 0.3 [a]

Mean values with different superscript ([a,b, c, d and x]) are significantly ($p < 0.05$) different

4.6. Comparative effect of vitamin C and E on Motility, Viability, Morphological defects and Malondialdehyde Concentration of Chilled Turkey Semen

The comparative mean motility, viability and MDA concentration of indigenous turkey tom semen preserved at refrigeration temperature (4^0C) is as shown in Table 4.2, 4.3, 4.4 and 4.5, respectively. Comparing the mean sperm motility values within the preservation time and between the various treatments, it was observed that at 0 hours, there was no significant difference ($p > 0.05$) in the mean spermatozoa motility of T0, T1, T2 and T3.The mean sperm motility at 0 hour post dilution was significantly ($p >0.05$) higher than the mean semen motility of T0, T1, T2 and T3 at 3, 6, 9, 12, 24 and 48 hours, respectively. At 3 hours of preservation, there was no significant ($p >0.05$) difference in the mean semen motility of T0 and T1, T0 and T3, T1 and T2, T1 and T3. However, there was a significant ($p <0.05$) difference in the mean sperm motility between T0 and T2 also T2 and T3. At 6 hours of preservation, there was a significant ($p <0.05$) difference between the mean sperm motility of T0 and T2. There was also a significant difference between T1 and T2 and between T2 and T3. The difference between T2 and T3 was significantly ($p <0.05$) higher than between T0 and T2 and also T1 and T2. There was no significant ($p >0.05$) difference in the mean motility values between T0 and T1, T0 and T3 and between T1 and T3. At 9 hours of preservation, the mean sperm motility values were significantly ($p >0.05$) different between T0 and T2, T1 and T2 and also between T2 and T3. The difference in mean spermatozoa motility was significantly higher between T0 and T2 than T2 and T3 and also between T1 and T2. There was no significant ($p >0.05$) difference between T0 and T1, T0 and T3 and also between TI and T3. At 12 hours of preservation, there was a significant ($p <0.05$) difference in mean semen motility between T0 and T2, T1 and T2 and also between T2 and T3. The difference between T0 and T2 was significantly (p

<0.05) higher than between T1 and T2 and also between T2 and T3. There was no significant (p>0.05) difference in the mean sperm motility between T0 and T1, T0 and T3 and also between T1 and T3.The mean sperm motility at 24 hours of preservation showed a significant (p<0.05) difference between T0 and T2, TI and T2 and also between T2 and T3. There was no significant (p>0.05) difference between the mean sperm motility of T0 and T1, T0 and T3 and between T1 and T3. There was no significant (p>0.05) difference in the mean sperm motility between the various groups at 48 hours of preservation. Comparing the mean sperm motility between the various groups and preservation time, the mean sperm motility of T0 decreased steadily within the first 12 hours of preservation after which there was a drastic decrease to 24 hours of preservation. The mean sperm motility of T0 decreased significantly (p <0.05) from 91.70% to 38.33% within the first 12 hours of preservation with an average decrease rate of 13% per 3 hours. The decrease was more drastic beyond 12 hours to 24 hours of preservation (38.33% to 0.00%). The mean sperm motility of T1 decreased significantly (p <0.05) from 81.70% to 46.67% within the first 12 hours of preservation, with an average decrease rate of 8.8%, then decreased more drastically for the next 12 hours (46.6% to 0.00%). The mean sperm motility of T2 decreased significantly (p<0.05) from 93.3% to 65.0% within the first 12 hours after preservation with an average decrease rate of 7.0% per 3 hours and with a more gradual decrease from 65% to 55% within the next 12 hours. The mean sperm motility of T3 decreased significantly (p<0.05) from 85.00 to 50.00% within the first 12 hours of preservation with an average decrease rate of 8.75% per 3 hours and a significant (p<0.05) decrease from 50.00% to 10.0% within the next 12 hours. Generally the average rate of decrease in spermatozoa motility for T1 (8.8%) and T3 (8.75%) was not significantly (p>0.05) different for the first 12 hours but was significantly (p<0.05) higher than the average rate of decrease of T2 (7.0%).

The rate of decrease of spermatozoa motility of T0 (13%) was significantly ($p<0.05$) higher than T1, T2 and T3.

Result comparing the mean sperm viability within the various treatment and preservation time showed that at 0 hour post-dilution, there was no significant ($p >0.05$) difference between the mean sperm viability of T0(94.6%), T1(93.0%), T2(92.3%) and T3(90.3%). At 3 hours of preservation, there was a reduction in the mean per cent viability however, there was no significant ($p >0.05$) difference in the mean viability result between T0 and T1, T0 and T3, T1 and T2, T1 and T3 and between T2 and T3. There was a significant ($p <0.05$) difference between the mean viability value between T0 (81.6%) and T2 (89.7%). The mean sperm viability values at 6 hours of preservation was significantly ($p <0.05$) lower than at 3 hours of preservation. There was significant ($p <0.05$) difference between the mean sperm viability of T0 (61.7%) and T1 (75.0%), T0(61.7%) and T2(82.0%), T0(61.7%) and T3(78.0%) also between T1(75.0%) and T2(82.0%). However, there was no significant ($p >0.05$) difference between the mean sperm viability of T1(71.3%) and T3(78.0%) and also between T2(82.0%) and T3(78.0%). At 9 hours of preservation, there was a decrease in the mean sperm viability across the various groups; however, the reduction was not significantly ($p>0.05$) different between T0(55%) and T1(65%), T0(55%) and T3(69.7%), T1 and T2(71%), T1(65%) and T3(69.7%) and between T2(71%) and T3(69.7%). There was a significant ($p <0.05$) difference between the mean sperm viability of T0 and T2. At 24 hours of preservation, the mean sperm viability was lower across the various groups than obtained at 12 hours of preservation. There was no significant ($p >0.05$) difference between the mean sperm viability of T0 (10.3%) and T1 (19%), and also between T1 (19.0%) and T3 (25.0%). There was significant ($p <0.05$) difference between the mean sperm viability of T0(10.3%) and T2(41.3%), T0(10.3%) and T3(25.0%),

T1(19.0%)and T2(41.3%) and also between T2(41.3%) and T3(25.0). The difference in mean viability value between T0 and T2 was significantly ($p < 0.05$) greater than between T0 and T3, T1 and T2 also T2 and T3. At 48 hours of storage, the mean viability value was lowest across the groups. There was a significant ($p < 0.05$) difference between the mean sperm viability of T0 and T1, T0 and T2, T0 and T3, T1 and T2 and between T2 and T3. The mean viability value between T0 and T2 was significantly higher than values between T0 and T1, T0 and T3, T1 and T2 and between T2 and T3. There was no significant ($p > 0.05$) difference in the mean semen viability value between T1and T3. Generally there was a consistent decrease in the mean viability values with storage time. The result also shows a consistent significant ($p < 0.05$) difference in the mean viability value between T0 and T2 for the first 12 hours of storage, and this significant difference was higher than obtained between the other groups.

Results from the morphological defects showed a general increase in spermatozoa morphological defects across the treatment groups and control over the storage time. There was no significant ($p < 0.05$) difference in the mean sperm defects across the treatment groups and control immediately after dilution (0 hours). After 3 hours of storage, there was no significant ($p < 0.05$) difference between the mean sperm defects in the treatment group and control. After 6 hours of preservation, the sperm morphological defects of T2 and T3 were lower ($p < 0.05$) than other groups. The sperm defects in T1 was lower than the control (T0). The sperm morphological defects in the treatment groups (T1, T2 and T3) were lower ($p < 0.05$) than obtained in the control (T0) after 9 hours of storage . The defects in T2 was significantly lower than T1. There was no significant ($p < 0.05$) difference the mean sperm defects between T1 and

T3 and T2 and T3. After 12 hours of storage, the sperm defects in the treatment group (T1, T2 and T3) were lower ($p < 0.05$) than the control (T0). There was no difference ($p > 0.05$) in the mean sperm defects between the treatment groups (T1, T2 and T3). At 24 hours post dilution, the mean sperm defects of T1, T2 and T3 were significantly ($p < 0.05$) lower than the control (T0). There was no significant ($p < 0.05$) difference between the mean sperm defects of T1 and T3 and also between T2 and T3. At 48 hour post dilution, the mean sperm defects of T1, T2 and T3 were significantly ($p < 0.05$) lower than the control (T0). There was no significant difference in the mean sperm defects between T1and T3 and also between T2 and T3. However, the mean sperm defect of T2 was lower than the defects in T1 after 48 hours of preservation.

Results from the MDA assay revealed a general increase in MDA concentration over the storage time. However, this increase was not consistently significant between most of the treatment groups. At 0 hours of storage, there was a significant ($p <0.05$) difference in the mean MDA concentration between T0 and T1, T0 and T2 and also between T0 and T3. The difference in mean MDA concentration between T0 and T2 was significantly ($p <0.05$) higher than between T0 and T1 and also between T0 and T3. There was no significant ($p >0.05$) difference in the mean MDA concentration between T1 and T2, T1 and T3 and also between T2 and T3. At 3 hours of storage, there was no significant ($p> 0.05$) difference in the mean MDA concentration between T0 and T1, T1 and T2, T1 and T3, and also between T2 and T3. There was a significant ($p <0.05$) difference between the mean MDA concentration of T0 and T2 and also between T0 and T3. At 6 hours of storage, the mean MDA concentration values were higher than values obtained after 3 hours of storage. However, there was a significant ($p <0.05$) difference between the mean MDA concentration across the groups (between T0 and

T1, T2, T3). At 9hours of storage, there was significant ($p < 0.05$) difference in the mean MDA concentration between T0 and T1, T0 and T2 and between T0 and T3. However, there was no significant ($p > 0.05$) difference between the mean MDA concentration between T1and T2, T1 and T3 and between T2 and T3. At 12 hours of storage, there was a significant ($p < 0.05$) difference in the mean MDA concentration between T0 and T1, T0 and T2, and between T0 and T3. There was no significant ($p > 0.05$) difference between T1 and T2, T1 and T3 and between T2 and T3. After 24 and 48 hours of storage, there was no significant ($p > 0.05$) difference between the mean MDA concentrations across the group. However, the mean MDA concentration was higher in all the groups than values obtained after 12 and 24 hours of storage, respectively.

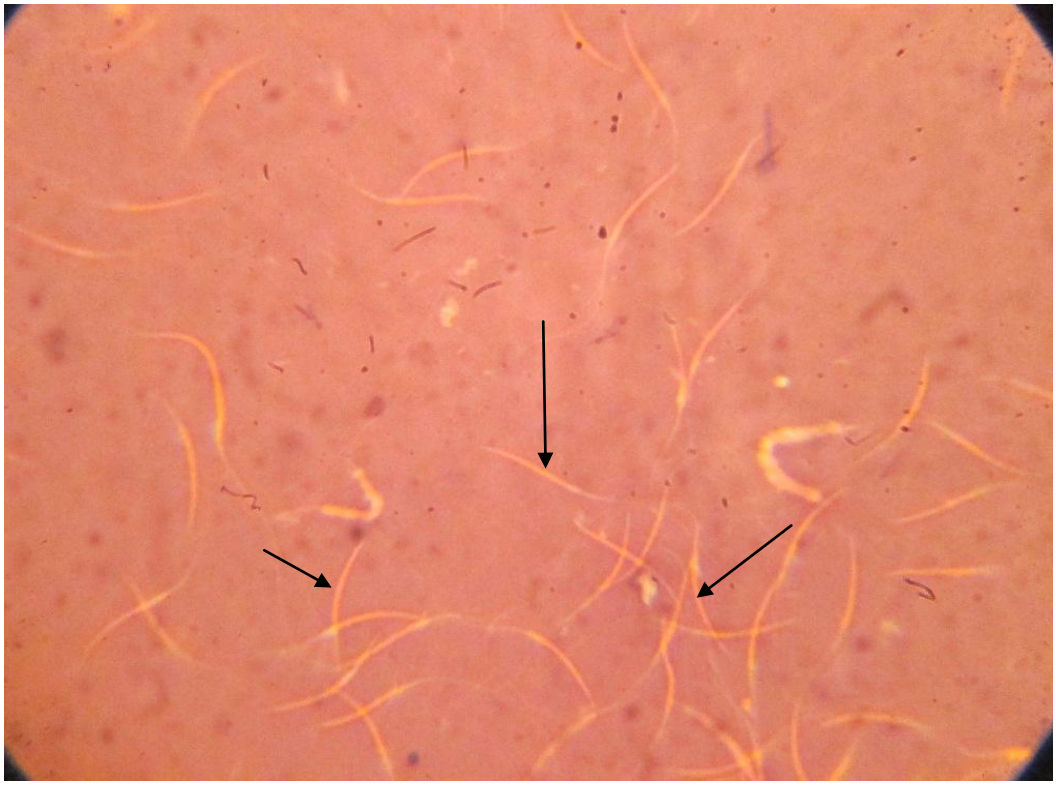


Plate X. Photomicrograph slide showing live spermatozoa (Black arrows)

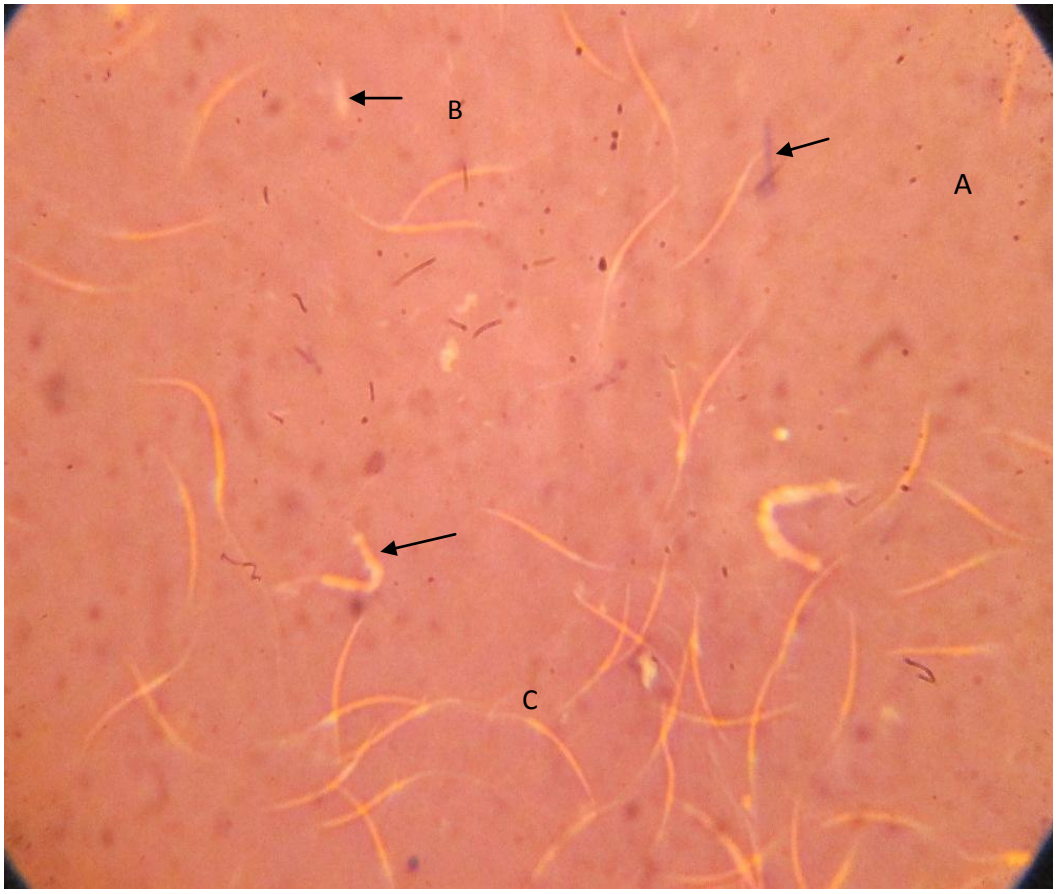


Plate XI: A photomicrograph showing some spermatozoa abnormalities (A-Coiled tail, B- Loose head and C- Bent mid-piece)

CHAPTER FIVE

DISCUSSION

The mean semen volume range of indigenous turkey toms (0.17-0.24 ml) from this study was lower than the acceptable range limit of 0.25-0.35 ml and 0.26-0.35 ml for toms reported by Burke (1984) and Bakst (1990). The overall mean volume (0.19±0.02 ml) from this study was lower than 0.5 ml in cocks reported by Hafez (1995) and also below the acceptable range of 0.25-2 ml presented by Cerolini *et al.* (2003) for chickens. Variation in ejaculate volume could be attributed to breed difference, season, age, frequency of semen collection and level of nutrition (Bakst and Cecil, 1992; Kelso *et al*., 1996; Van Wambeke, 1996). Etchu *et al*. (2013) also reported that sperm output in toms varies with breed, nutrition and season. The mean semen volume yield of 0.19 ± 0.01 from the indigenous turkey toms used in this study was similar to the result reported by Ngu *et al*. (2014). The semen volume of the indigenous turkey toms was however lower than the yield from exotic turkey toms (0.35 ± 0.05 ml) (Ngu *et al,* 2014). Kozłowski *et al*. (2004) and Glogowski *et al*. (2004) also reported that semen volume and concentration were a function of the turkey strain and they concluded that the Big-6 strain yielded higher semen volume and concentration when compared to other strains. It seems therefore, that the lower volumes recorded in the current study could be as a result of type of breed, season and age of toms used.

The semen colour of indigenous turkey toms from this study (milky-creamy) correlates with reports of Etches (1998) who reported that any deviation in colour from milky to creamy colour of poultry semen could mostly be attributed to contaminants. The mean spermatozoa concentration in this study 6.66 ± 0.66 x 10^9/ml was higher than values reported for indigenous turkey toms by Zahraddeen *et al.* (2005), and was lower than

the value reported for exotic turkey flocks by Idi (2000) and Zahraddeen *et al*. (2005). Ngu *et al.* (2014) reported that there was no significant difference of sperm concentration between indigenous and exotic toms. Mohan *et al*. (2013) reported a lower sperm concentration (3.27± 0.14 $x10^9$ /ml) in guinea fowls. The variation in sperm concentration could be influenced by breed, nutrition, season and method of semen collection (Butswat *et al.*, 2002).

Motility, expressed as the percentage of spermatozoa moving through the power of their tail is used as an indicator of fertility. The mean motility value (90.50 ± 1.01) from this study was within the established normal range proposed by Sexton (1981) who reported a mean motility of 80% and above as excellent. The mean motility agrees with the values reported by Taras *et al*. (1997) and Zahraddeen *et al*. (2005) who both reported a mean motility of above 80% for exotic and indigenous turkey toms respectively. The later went further to show that there was no significant difference in the mean motility between indigenous and exotic turkey stock.

The pH of indigenous turkey semen obtained from this study (6.85± 0.05) was within the range reported for poultry by Lake and Ravie (1981) and Etches (1998). A lower semen pH can be attributed to contamination mostly by urine during collection (Bakst and Long, 2010). The percent live spermatozoa which is used as an index of viability measurement from this study (90.54 ± 1.09%) was slightly higher than the value reported by Long and Kramer (2003) who reported a percent live of 86.20% in fresh semen of exotic turkey toms. The per cent live spermatozoa from this study were also higher than the value reported for indigenous toms by Ngu *et al.* (2014). Despite the variation between the per cent live spermatozoa from this study and from previous reports, the per cent live spermatozoa exceeded the minimum score of 70-80% required for insemination.

The per cent mean deformed spermatozoa from this study (7.77 ± 0.65%) was lower than the value (14.36 ± 1.17%) reported for indigenous toms by Ngu *et al*. (2014). Zahraddeen *et al*. (2005) also obtained a higher percent of deformed spermatozoa from indigenous turkey toms. Other studies carried out on other poultry species revealed significant variation in the per cent deformed spermatozoa. For example, Butswat *et al*. (2002) reported mean abnormal spermatozoa of 33.40 ± 0.76% from indigenous guinea fowls. He also reported a lower mean of per cent deformed spermatozoa from exotic guinea fowls. Gbadamosi and Egbunike (1999) also obtained means of 10.66 ± 0.24 and 12.80 ± 0.30% for total abnormal sperms of exotic and local cocks.

Usually sperms deformities indicate disturbances of spermatogenesis and this could be attributed to age, nutrition and pollution (Bah *et al*., 2001). Improper handling of semen during processing has also been incriminated for semen deformities (Ajayi *et al*., 2011). Ajayi *et al*. (2011) reported that spermatozoa deformities did not show any strain or breed correlation in indigenous cocks from a study conducted on various strains of indigenous cocks.

The MDA concentration in fresh semen samples from indigenous turkey toms was similar with the reports of Long and Kramer (2003) which demonstrated that some lipid peroxidation naturally occurs within the male reproductive tract and that this level of lipid peroxidation does not impair fertility in turkeys. Blebois *et al*. (1993) also reported that peroxides were present at ejaculation in roosters. The MDA concentration from this study was in agreement with the findings of Wishart (1984) and Long and Kramer (2003) which demonstrated that lipid peroxidation differs in individual male birds. The initial MDA concentration of indigenous turkey semen from this study (0.72-

0.74 µl/ml) was lower than the values (0.92-1.36 µl/ml) reported by Long and Kramer (2003). It was also lower than the values reported in roosters by Blebois *et al*. (1993). The discrepancy in MDA concentration may be attributed to the breed of birds involved, the age of the birds and the technique used in the measurement of MDA. Donoghue and Donoghue (1997), reported that MDA production was 10-folds higher in toms after 26 weeks of semen production compared to males after 2 weeks of semen production. Long and Kramer (2003) obtained higher MDA values from turkey semen using a more sensitive technique which measures total MDA (free and protein-bound) by the formation of a stable chromatophore. The TBA- MDA technique used in this current study was said to be less accurate because it measures only free MDA and other TBA reactive substances can interfere with the MDA-TBA reaction.

There was no significant difference in the mean semen motility between the various treatment groups and the control at 0 hours post-dilution. This was in agreement with the report of Donoghue and Donoghue(1997) in toms . The motility results decreased across the groups with increase in storage time but the decrease in per cent motility was more drastic for the control (T0) and T1 (VIT C) which recorded a motility of 0% at 24 hours of storage. This result was in agreement with the report of Donoghue and Donoghue (1997) which stated that the addition of vitamin C to semen diluents did not produce any difference in sperm motility over the control during the storage period of study. Other authors reported a similar observation when vitamin C was supplemented to semen diluents during *in vitro* studies. Spallanzani (1989) reported that vitamin C had no effect on the motility of human spermatozoa. Similar result was reported in rams (Bathgate *et al*., 2008) and horses (Dukelow and Graham, 1962; Aurich *et al*., 1997). Furthermore, vitamin C was reported to be a powerful antioxidant when peroxyl

radicals are located in the aqueous phase but a poor scavenger for ROS within the lipid membrane (Burton and Ingold, 1986). Beconi *et al*. (1993) observed that vitamin C could function as an antioxidant at lower concentration and as a pro-oxidant at a higher concentration. At the concentration used in this study, vitamin C did not yield a better motility values over the control for 24 hours of storage. There was a more gradual reduction in semen motility in group T2 (VIT E) and T3 (VIT C+E) over a more prolonged period of storage. This finding was in agreement with the reports of Donoghue and Donoghue (1997) and Blebois *et al*. (1993). The possible reason for the gradual decrease in semen motility for T2 and T3 may be attributed to their solubility and distribution of the antioxidants within and between the semen components.

Vitamin E has a larger distribution within the plasma membrane of spermatozoa and its lipid solubility allows it to suppress peroxidative damage across the plasma membrane. Vitamin E supplemented semen (T2) displayed a slower rate of mean motility decrease and was followed by T3 (VIT C+E). This result is in agreement with the reports of Al-Daraji (2002), Donoghue and Donoghue (1997) and Tabatabaei *et al.* (2011). However, Blebois *et al.,* (1993) reported that the addition of vitamin E to cock semen did not show any difference in motility between the control and treated group when the semen was preserved for 24 hours in vitro. The combination of vitamin C and E showed slower motility decrease over the period of storage and this result could be attributed to the synergistic action between vitamin C and E as reported by Whitehead and Keller (2003). Although vitamin E was reported to inhibit ROS generation, the availability of vitamin C increases the antioxidant activity of vitamin E by regenerating the reduced vitamin E radical (alpha-tocopheroxyl radical) back to alpha tocopherol (Wenk *et al*.,

2000) for continuous antioxidative action or it can be stored (Parker *et al*., 1979; Eicheinberger *et al*., 2004).

The viability results from this study showed that antioxidant supplementation of turkey semen diluents improved the viability of stored turkey semen over the control, however, none of the antioxidants was able to protect against viability loss during the period of study. There was no significant difference in viability between the treatment groups and the control immediately after dilution. However, there was a progressive loss of viability across the treatment groups and control over the time period of the study. This report was in agreement with the report of Al-Daraji (2002) who reported an improvement in semen quality of roosters supplemented with vitamin C and E. The result from this study was also in agreement with the reports of Ceylan and Serin (2007) who reported an improvement in the viability of dog semen supplemented with ascorbic acid. The possible better viability result of vitamin C (T1) compared to the control could be attributed to the efficiency of ascorbic acid as an antioxidant which scavenges ROS thus protecting sperm membrane against lipid peroxidation. The result from this study showed that vitamin E (T2) had a better viability compared to vitamin C (T1), a combination (T3) and control (T0) over the storage period. The result from vitamin E supplementation from this study is supported by the earlier report of Donoghue and Donoghue (1997). Similar results were also reported by Cerolini *et al*. (2006); Tabatabaei *et al.* (2011) who both observed that supplementing semen extender with vitamin E improved spermatozoa quality parameters including motility and viability of rooster semen. Similar reports were also documented in boars, rams, human and rats by Brezezinska-Slebodzinska *et al*. (1995); Cerolini *et al*., 2000 Luo *et al*. (2004); Liu *et al*. (2005), Akiyama (1999) and Sonmez *et al.* (2005), respectively. The maintainance of semen viability by addition of vitamin E to the semen diluents in this present study

could be attributed to the high lipid solubility and membrane-stabilizing antioxidant effect of vitamin E within the cell membrane as proposed by Tabatabaei *et al.* (2011).

The viability preservation result obtained from a combination of vitamin C and E (T3) in comparison to vitamin C (T1) and the control (T0) from this study was in agreement with the findings of Shireen *et al.* (2008) who observed a synergistic effect between Vitamin C and E and showed that the combination of vitamin E and C enhanced the activities of other antioxidant enzymes significantly. He also reported that vitamin C and E helped to reduced the risk of chronic diseases and cells damage related to oxidative stress. Several studies have shown that the association of ascorbic acid and their derivatives with vitamin E enhanced the antioxidant capability of both the two vitamins (Madhavi *et al*., 2009). In studies with human subjects, vitamin C supplementation increased plasma lipid standardized α vitamin E (Hamilton *et al*., 2000). Other contrary reports have also been observed by some researchers.

Donoghue and Donoghue (1997) reported that the addition of ascorbic acid did not improve mentainance of viability during chilled storage of turkey semen. Long and Kramer (2003) also reported that vitamin E supplementation did not improve turkey sperm motility and viability after 24 hours of cold storage. The different experimental designs might have contributed to the discrepancy in the result.

The lower spermatozoa morphological defects obtained in the treatment groups over the storage period as against the control group were in accordance with reports of Aurich *et al*. (1997); Yousef *et al*. (2003) and Tabatabaei *et al.* (2011) who reported that the supplementation of the extended semen with antioxidant vitamins reduced the morphological defect rates of spermatozoa during 24 hours of storage at 4^0C. From this study vitamin E treated group had lesser morphological defects over the storage

period. This finding was in accordance with the reports of Behne *et al*. (1996); Kohrle *et al*. (2005) ; Olsen (2004) and Tabatabaei *et al*. (2011) who reported that the supplementation of semen with vitamin E reduced the morphological defects rate over a 24 hour of storage. Donoghue and Donoghue, 1997 also reported that the addition of vitamin E to *in vitro* stored turkey semen resulted in the maintainace of sperm membrane integrity. The positive result from vitamin E supplementation to turkey semen diluents could be attributed to the inhibition of lipid peroxidation and the mentainance of semen plasma membrane integrity by Vitamin E (Somnez *et al*., 2005).

Another study reported that the quality of stored turkey semen was not improved by the addition of vitamin E (Long and Kramer, 2003). The group supplemented with combined vitamin C and E performed better than vitamin C alone in reducing the rate of sperm morphological defect rates during *in vitro* storage. This finding agrees with an earlier report by Surai, 2002: Long and Kramer, 2003 who stated that there was synergism between vitamin C and E as shown by their mutual activity. They further hypothesized that there is a negative influence of lipid peroxidation on sperm energetic affecting turkey sperm fertilizing ability. They concluded that the addition of vitamin E alone was not enough to prevent lipid peroxidation of spermatozoa plasma membranes. The findings from this study are also supported by an earlier report by Maia *et al*. (2009:2010). They stated that the continuous free radical scavenging role of vitamin E is dependent on its reduction to its active form by vitamin C.

The group supplemented with vitamin C alone performed better than the control with lesser morphological defect rate compared to the control. This finding was in agreement with earlier reports by Azawi and Hussein (2013). They concluded that the addition of vitamin C did not only improve the viability but also maintained acrosome and

membrane integrity of ram semen for over 120 hour of preservation. Other similar reports were presented by Singh *et al.* (1996) in buffalo; Maia *et al*. (2010) in ram semen and Hughes *et al*. (1998) in human semen. Ceylan and Serin, 2007 reported the beneficial effects of ascorbic acid supplementation to the semen of dogs. He showed that vitamin C protected the sperm membrane integrity and this was attributed to the fact that vitamin C is an efficient antioxidant and a scavenger of free radicals (Dawson *et al*., 1992). Vitamin C was also shown to protect the activities of superoxide dismutase and regenerates other antioxidative systems (Beconi *et al*., 1993). However, in another study, the addition of ascorbic acid did not improve the quality of stored turkey semen (Donoghue and Donoghue, 1997).

The MDA concentration result across the treatment groups and control from this study was in agreement with the proposal of Long and Kramer (2003), who demonstrated that lipid peroxidation in poultry ejaculate occurs during aerobic preservation in a time and temperature dependent manner. Neither the aerobic condition nor the refrigeration temperature (4^0C) was able to prevent lipid peroxidation from this study. Similar reports were demonstrated by Cecil and Bakst (1993); Blebois *et al*. (1993) in turkey and roosters respectively. Data from this study shows that individual toms differ in the degree of lipid peroxidation, producing different amount of MDA at ejaculation. This finding was in agreement with the findings of Long and Kramer, (2003). The result from this study also showed that the treated groups performed better in reducing lipid peroxidation as compared to the control. However, none of the treatments could prevent lipid peroxidation. To the best of my knowledge only few research results have been documented on the effect of antioxidant supplementation on lipid peroxidation in poultry. T0 (control) recorded the highest MDA concentration over the 48 hour period

of study. This value corresponds to a higher level of lipid peroxidation in the control as against the treatment groups. T1, T2 and T3 all recorded lower MDA concentration over the study period with T2 having the lowest MDA level. This finding suggest that Vitamin E and a combination of Vitamin C and E performed better in reducing lipid peroxidation in turkey semen during *in vitro* preservation at 4^0C. Different authors have presented inconsistent results on the effect of Vitamin C and E on lipid peroxidation. Das *et al.* (2009) reported a negative correlation between seminal ascorbic acid and MDA concentration. Similar result was reported by Fanaei *et al*. (2014) and Nouri *et al*. (2008). Vitamin E was reported to have reduced lipid peroxidation in boar semen (Cerolini *et al*., 2000). Similar result was reported in rams (Upreti *et al*., 1997). Vitamin E was reported as ineffective in reducing lipid peroxidation in turkey semen under aerobic condition (Long and Kramer,2003). Ball and Vo (2002) reported similar results in horses. The lower MDA result obtained from this study (T2) could be attributed to the free radical quenching action of vitamin E, thus acting as a major chain breaking antioxidant (Bansal and Bilaspuri, 2009) . The result obtained from the combination of vitamin C and E from this study was in line with the report of Sikka (2004) who observed an MDA decrease in vitamin C and E treated group as against the control. This finding from this current study could be attributed to the synergy of antioxidative properties of vitamin C and E in the prevention of lipid peroxidation in sperm cells.

CHAPTER SIX

CONCLUSIONS AND RECCOMENDATIONS

6.1 Conclusions

The result from this study showed that antioxidant supplementation of turkey semen extenders helped to preserve semen motility, viability and reduced the rate of morphological abnormalities during *in vitro* semen storage at 4^0C. Turkey semen supplemented with vitamin E performed better than vitamin C and a combination of vitamin C and E in maintaining turkey semen motility (55.0 ± 2.9 %), viability (41.3 ±1.4 %), reduced rate of morphological defects (24.2± 0.3 %) and a lower MDA level (2.5±0.2) after 24Hr of storage at 4^0C .

Vitamin E or a combination of vitamin C and E could be used to preserve turkey semen for 24 hours at 4^0C.

6.2 Reccomendations

Turkey toms intended for use in AI or breeding should be assessed for semen quality prior to use.

Semen not intended for immediate use in AI should be extended and supplemented with vitamin E and such extended semen should not be preserved longer than 24 hours before use.

Further studies should be carried out to study the fertility using semen supplemented with antioxidants for AI.

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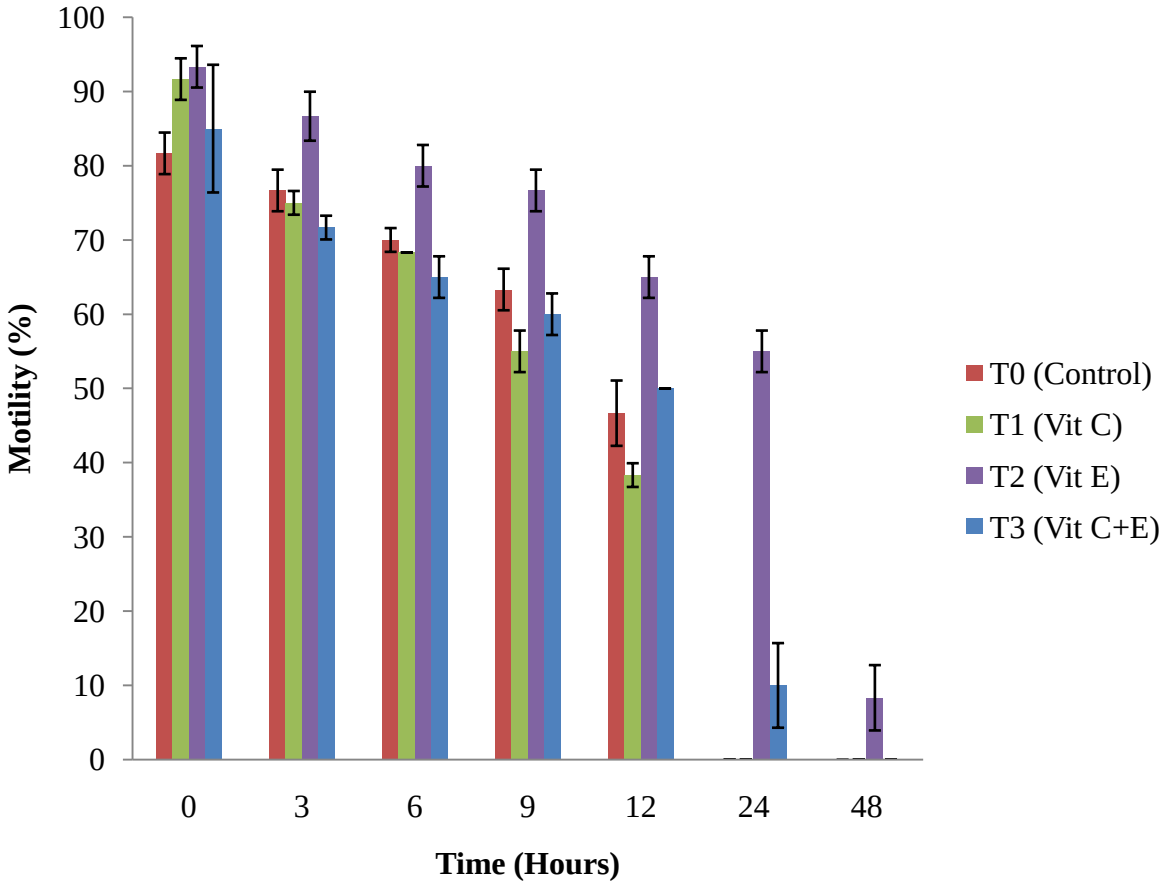
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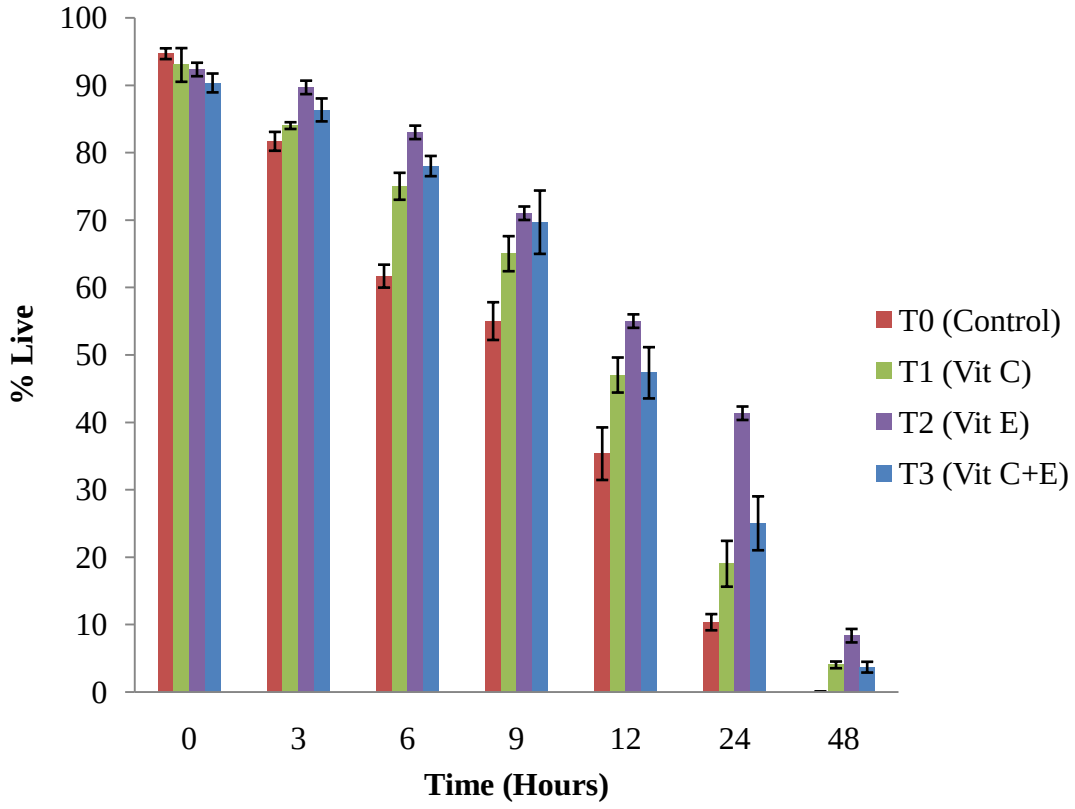
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APPENDICES

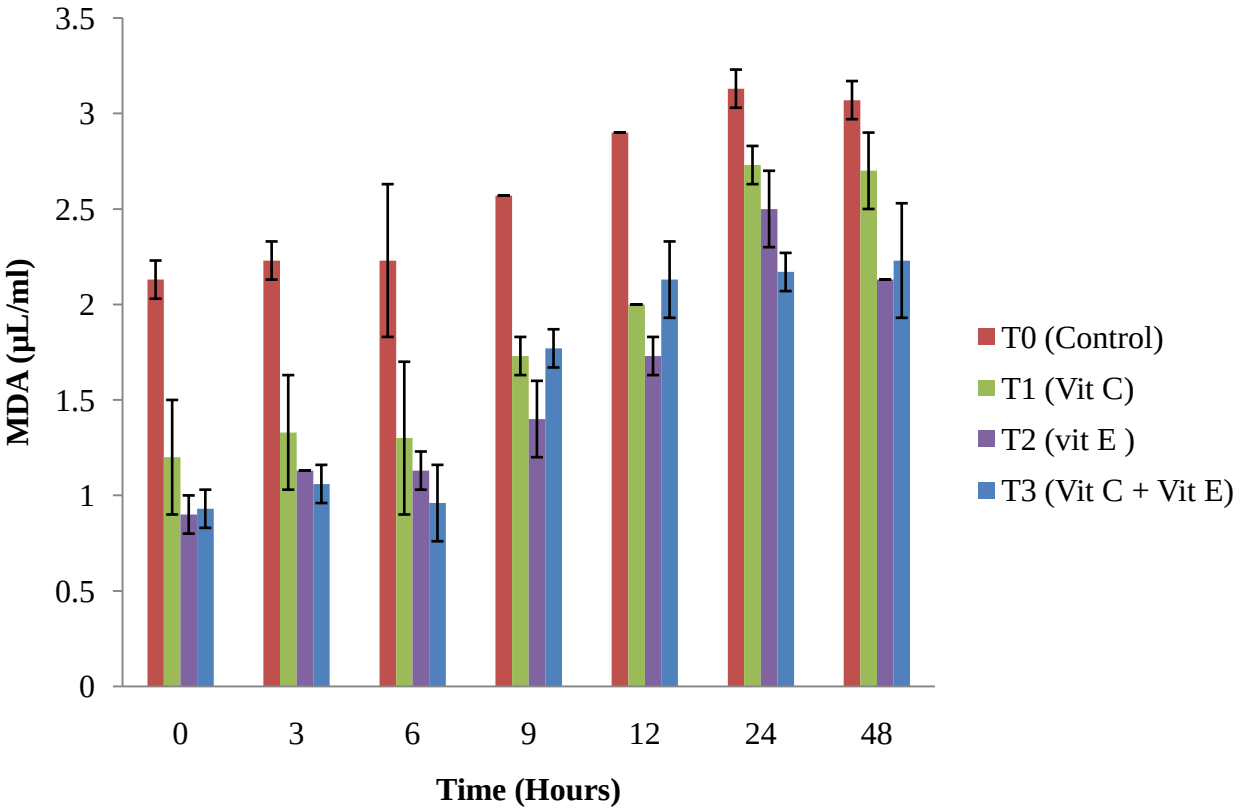
Appendix I: Effect of vitamin C, E and their Combination on Motility of Chilled Turkey Semen



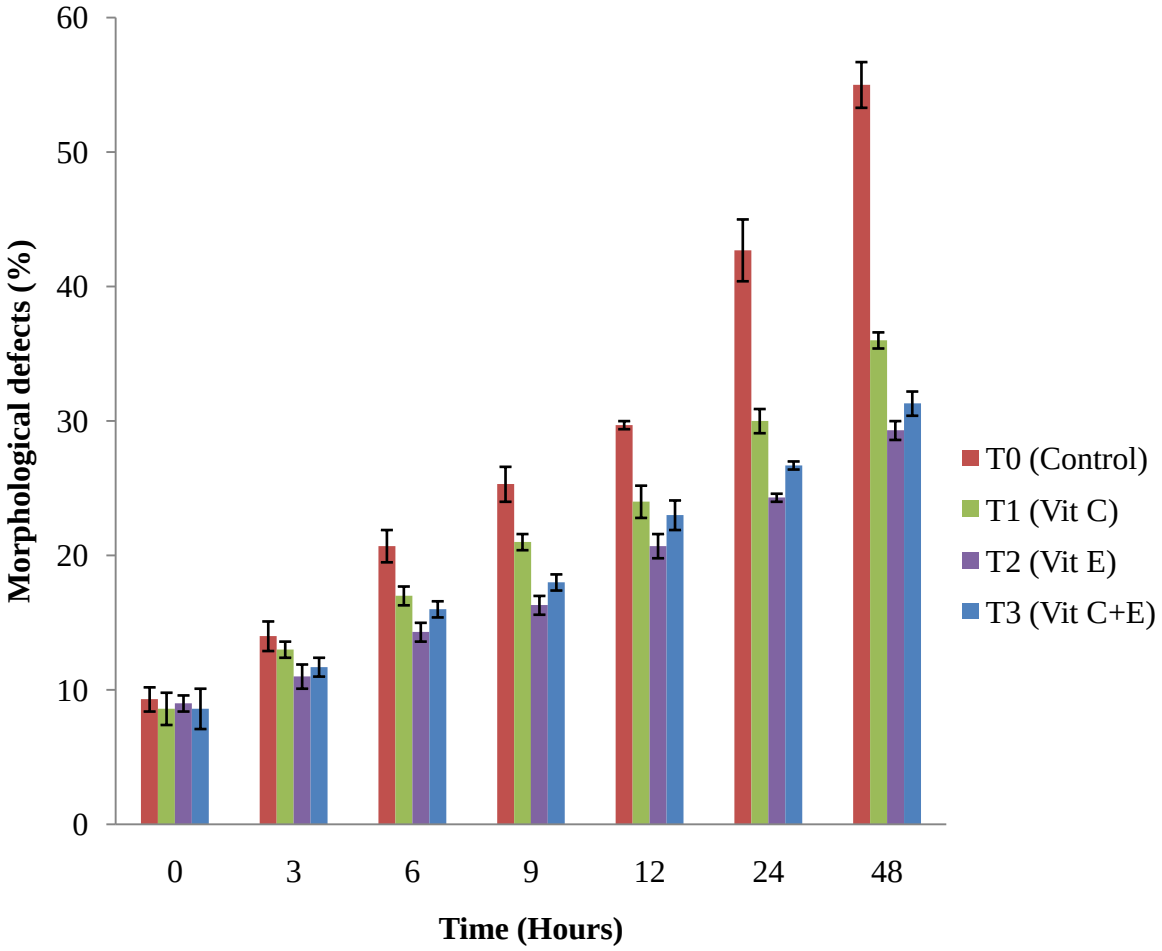
Appendix II: Effect of vitamin C, E and their Combination on Viability of Chilled Turkey semen



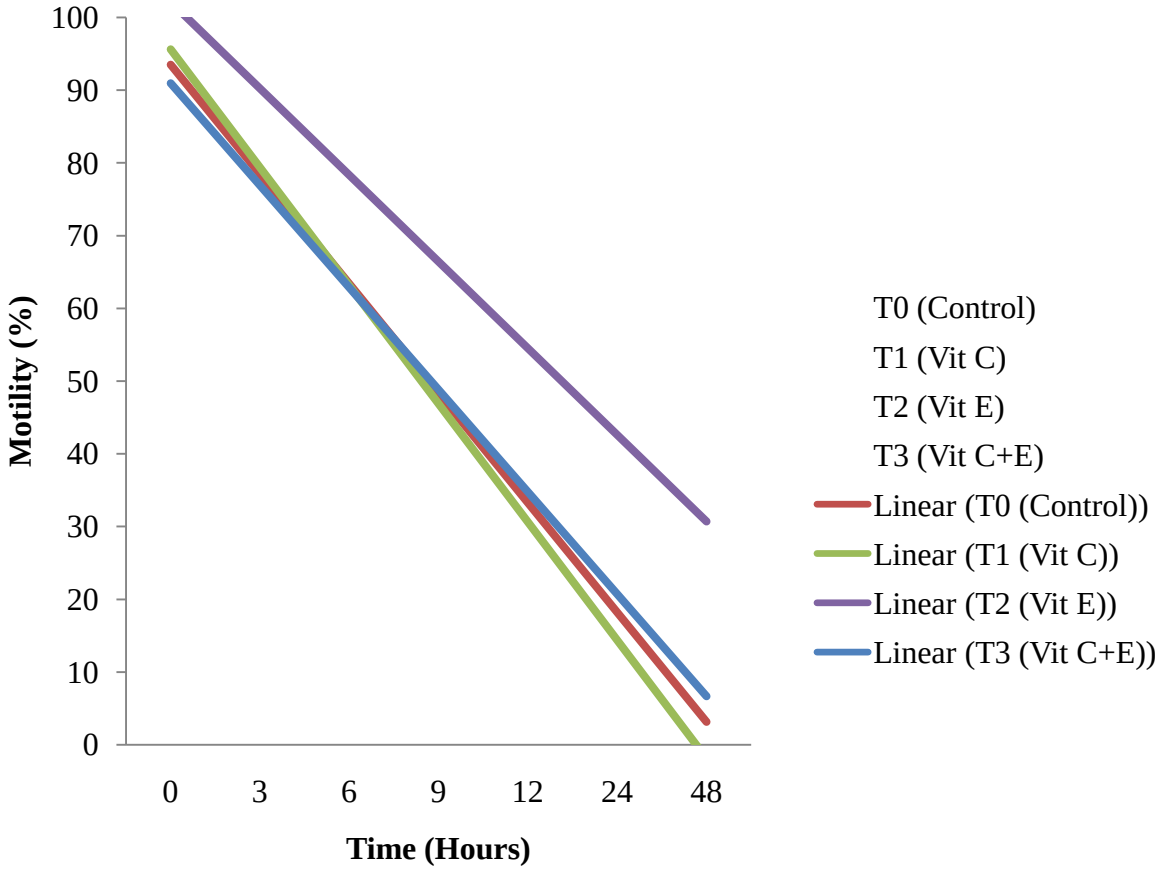
Appendix III: Effect of vitamin C, E and their Combination on MDA Concentration in Chilled Turkey Semen



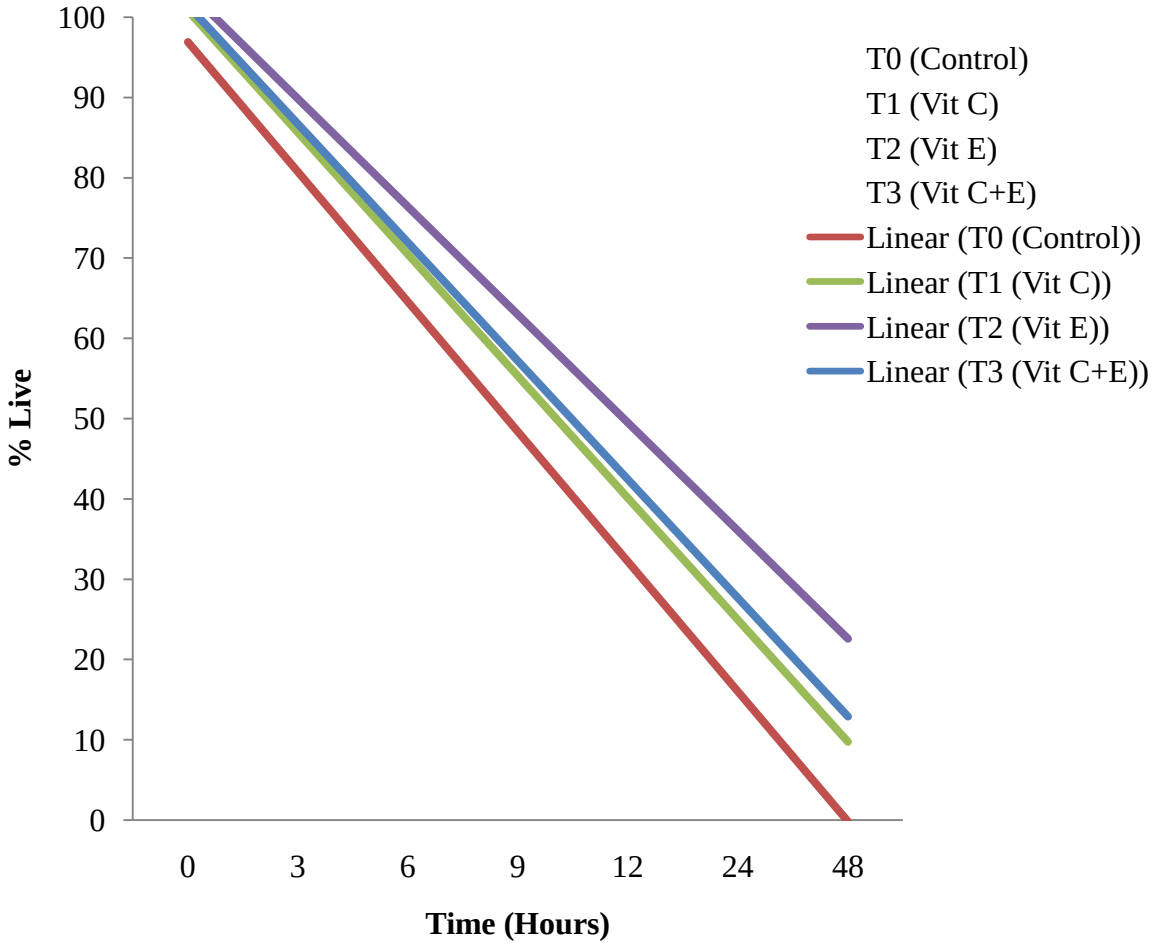
Appendix IV: Effect of vitamin C, E and their combination on morphological defects in chilled turkey semen



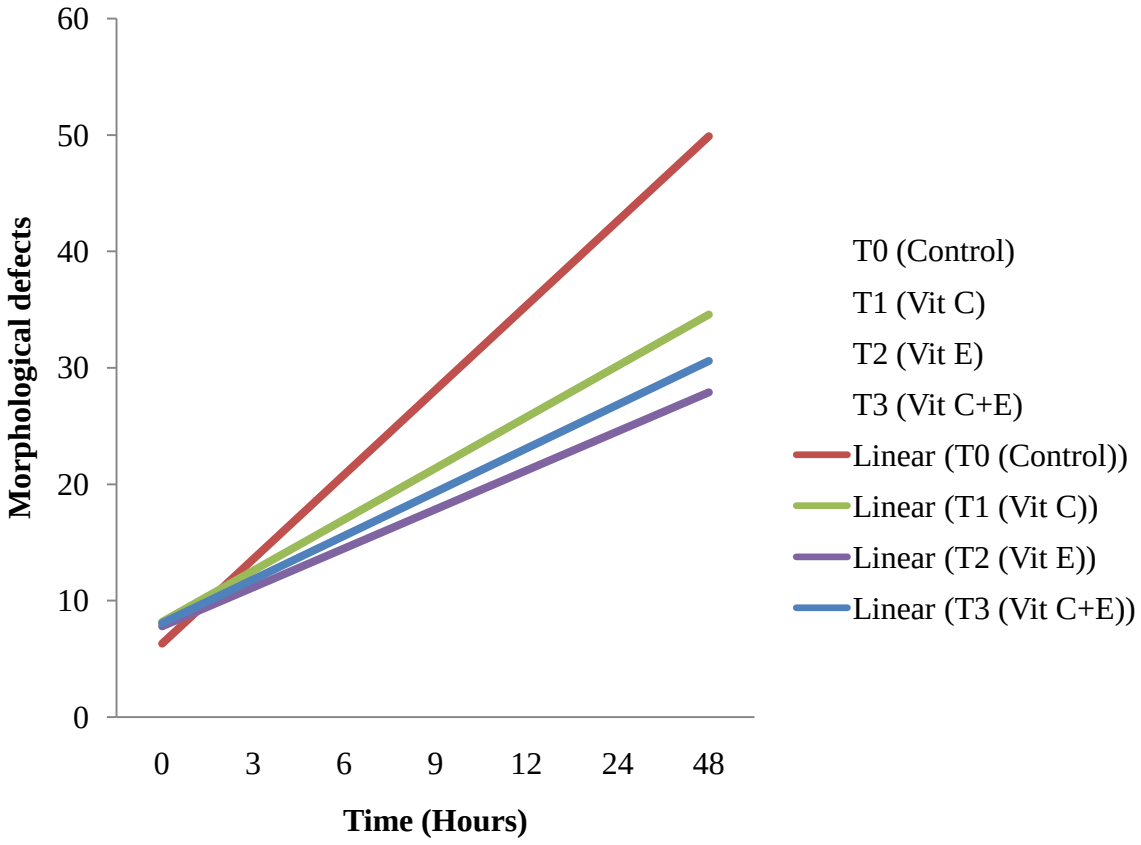
Appendix V: Effects of vitamin C, E and their Combination on the rate of Motility loss of Chilled Turkey Semen



Appendix VI: Effects of vitamin C, E and their Combination on the rate of Viability loss of Chilled Turkey semen



Appendix VII: Effect of vitamin C, E and their combination on the rate of morphological defects in chilled turkey semen



Appendix VIII: Effect of vitamin C, E and their Combination on the rate of MDA formation in Chilled Turkey Semen

