

**EFFECTS OF DIFFERENT AVIAN EGG YOLKS AND VITAMIN
C INCLUSION IN TRIS-SODIUM CITRATE EXTENDER ON
CHARACTERISTICS OF CHILLED BOVINE SEMEN.**

BY

JESSICA BAZACHAT GOJE

**DEPARTMENT OF ANIMAL SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA.**

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CHARACTERISTICS OF CHILLED BOVINE SEMEN.**

BY

**Jessica Bazachat GOJE,
B. Agric (A.B.U., Zaria)
MSc/Agric/20921/12-13**

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**DEPARTMENT OF ANIMAL SCIENCE, FACULTY OF
AGRICULTURE, AHMADU BELLO UNIVERSITY, ZARIANIGERIA.**

JULY, 2015

DECLARATION

I declare that the work in this project thesis entitled “**Effects of Different Avian Egg Yolks and Vitamin C inclusion in Tris-Sodium Citrate Extender on Characteristics of Chilled Bovine Semen**” has been carried out by me in the Department of Animal Science. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project thesis was previously presented for the award of another degree or diploma at this or any other institution.

JESSICA BAZACHAT GOJE

(Student Name)

.....

(Signature)

(Date)

CERTIFICATION

This project thesis entitled **“EFFECTS OF DIFFERENT AVIAN EGG YOLKS AND VITAMIN C INCLUSION IN TRIS-SODIUM CIRATE EXTENDER ON CHARACTERISTICS OF CHILLED BOVINE SEMEN”** by **JESSICA BAZACHAT GOJE** meets the regulations governing the award of the degree of Master of Science (Animal Science) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Signature:

Date.....

DR P. P. BARJE
Chairman, Supervisory Committee
National Animal Production Research Institute,
Ahmadu Bello University, Zaria

Signature:

Date.....

PROF P. I. REKWOT
Member, Supervisory Committee
National Animal Production Research Institute,
Ahmadu Bello University, Zaria

Signature:

Date.....

DR S. DURU
(Head of Department
Department of Animal Science
Ahmadu Bello University, Zaria

Signature:

Date.....

PROF A. Z. Hassan
Dean, School of Postgraduate Studies
Ahmadu Bello University, Zaria

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ABSTRACT

Two experiments were carried out to evaluate the effect of extenders made from the egg yolk of four avian species (chicken, guinea fowl, turkey and quail) and three inclusion levels of Vitamin C (0, 3 and 6mg/ml) of the best two egg yolk extenders (chicken and quail) on characteristics of bull semen at different storage periods (0, 24, 48 and 72 hours). Both experiments were replicated for five weeks using three Friesian× Bunaji bulls respectively. The result of the first experiment shows that egg yolks had significant effect ($P<0.05$) on motility, viability and total abnormalities of bovine spermatozoa. Chicken egg yolk Tris-sodium citrate extender had significantly higher ($P<0.05$) motility and viability with lower ($P<0.05$) total abnormalities after 72 hours. Turkey egg yolk extender had significantly lower ($P<0.05$) motile, viable sperm cells and higher ($P<0.05$) total abnormalities. Quail egg yolk extender had significantly lower ($P<0.05$) sperm abnormalities than turkey egg yolk extender though there was no significant difference in their motility after 72 hour. The result of the second experiment shows that the inclusion levels of Vitamin C on chicken and quail egg yolk extender had significant effect ($P<0.05$) on motility, viability, normal cells abnormalities of bull spermatozoa but no significant effect ($P>0.05$) on semen pH at 0, 24, 48 and 72 hours respectively. After 72 hours there was significant higher ($P<0.05$) motile, viable and normal sperm cells in the quail and egg yolk extenders with higher inclusion level of Vitamin C compared to their control. In conclusion, this experiment reveals that chicken egg yolk extender did best in cryopreservation of bull semen and is followed by quail egg yolk. Also inclusion of Vitamin C up to 6mg/ml in both chicken and quail egg yolk semen extenders maintained semen quality.

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ABBREVIATIONS

A.I	Artificial Insemination
ANOVA	Analysis of Variance
AV	Artificial Vagina
BHT	Butylated Hydroxytoluene
BSA	Bovine serum Albumin
BT	Bent Tail
CEYE	Chicken Egg Yolk Extender
cm	Centimeters
CSR	Cryopreserved Sperm Rates
CT	Coiled Tail
DMRT	Duncan Multiple Range Test
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonuclei Acid
E,g	Example
FAO	Food and Agricultural Organisation
FT	Free Tail
GEYE	Guinea Fowl Egg Yolk Extender
g	Gram

GPx	Glutathione Peroxidase
GR	Glutathione reductase
H ₂ O ₂	Hydroperoxide
HDL	High Density Lipoprotein
i.e	that is
IgY	Immunoglobulin Y
IVF	Invivo Fertilization
Kg	Kilogram
LDL	Low density Lipoproteins
LN ₂	Liquid Nitrogen
LOS	Level of Significance
LPO	Lipid Peroxidation
Mg	miligram
MI	millitre
MPD	MidPiece Droplet
NAPRI	National Animal Production Research Institute
NADH	Nicotinamide adenine dinucleotide
NS	Normal Sperm
HF	Holstein Freisian

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphotidylinositol
PS	Phosphatidylserine
PUFAs	Poly Unsaturated Fatty Acids
QEYE	Quail Egg Yolk Extender
ROS	Reactive Oxidative Stress
SAS	Statistical Analysis System
SCB	Sodium Citrate Buffer
SDO	Superoxide Dimutase
SE	Standard Error
SEM	Standard Error of Means
SPH	Sphigomyelin
TEYE	Turkey Egg Yolk Extender
⁰ C	Degree Celsius
%	Percentage

CHAPTER ONE

1.0 INTRODUCTION

FAO (2011) estimated the number of cattle in Africa during the period 2001 to 2010 to be twice the estimates for the years 1961–1970. The role of cattle in developing countries like Nigeria as a source of high-quality food, draft animals, and as source of manure and fuel cannot be over emphasized (Tadesse *et al.*, 2003). Cattle represent important contribution to household incomes (Seo and Mendelsohn, 2006), and in drought prone areas they can serve as an insurance against weather risk (Fafchamps and Gavian, 1997). As the demand for these animals and their products is constantly increasing around the world, the prospects for increasing the number and productivity of these animals need to be utilized (FAO, 2011). Some techniques of reproductive physiology have been applied to animal breeding for the achievement of faster genetic improvement. These techniques include artificial insemination, oestrus synchronization, induction of multiple ovulation, anti-steroid immunization, long term storage of gametes and embryo transfer (Gordon, 2004). Using Artificial insemination (AI) as the first biotechnology widely implemented in practice is important for selection and breeding of cattle (Gravance *et al.*, 2009). The process of AI involves semen collection, evaluation, processing, preservation and final introduction into the genitalia of an oestrous female (Thibier and Wagner, 2002). Semen that has undergone processing with the principle of preservation can be stored in temperatures as low as 5⁰C and -196⁰C. However, negative changes in sperm membranes in relation to storage time and the extender has been demonstrated (Watson, 2000^b; Frydrychová *et al.*, 2010). The use of chilled (liquid) semen has been said to be a cheap solution to the decline fertility of frozen semen and is more effective and efficient (Sri *et al.*, 2012) without the need for

liquid nitrogen and the incidence of fertility decline compared to frozen semen (Gadea *et al.*,2004).

Chilling semen can be prepared using extenders which are based on either ionic or non-ionic substances that prevent changes in osmolality and act as a buffer against changes in pH (Vishwanath and Shannon, 2000). Semen diluents (extenders) provide nutrients for spermatozoa during the storage period, allowing spermatozoa to be able to keep moving progressively. They are non-toxic to spermatozoa, contain buffer and able to protect the spermatozoa from cold shock during cooling or freezing processes (Sri *et al.*, 2012).

Chicken egg yolk has been used as a basic component of extenders for bull semen since 1939 (Amirat *et al.*, 2004) and still remain popular. Although the addition of egg yolk changes the composition of an extender, it's recommended because of the excellent protection, it offersto sperm cells (Celeghini *et al.*, 2008). Also, its wide availability (Sugulle *et al.*,2006), beneficial effects on sperm viability as a protectant of the plasma membrane and acrosome against cold shock during chilling or cryopreservation (Amirat *et al.*, 2004). The phospholipids, cholesterol and low density lipoproteins of chicken egg yolk specifically have been identified as the protective components (Anton *et al.*, 2006). However, the chemical composition of the egg yolk of different avian species varies particularly in terms of cholesterol, low density lipoproteins and phospholipid contents (Burriss and Webb, 2009). These may influence their effectiveness when chilling, freezing and post-thawing semen extenders (Bathgate *et al.*, 2006).

Sperm cells have a high content of unsaturated fatty acids in their membranes, while lacking a significant cytoplasmic component containing antioxidants (Hu *et al.*, 2010). Sperm cellsare very susceptible to lipid peroxidation by free radicals such as hydrogen

peroxide, superoxide anion, and hydroxyl radical, which could lead to the structural damage of the sperm membranes during the storage (Azawi and Hussein, 2013). Free radicals seek stability by “stealing” electrons from nucleic acids, lipids, and proteins which leads to the damage of cells (Yue *et al.*, 2010). Free radicals are mostly eliminated by antioxidant systems. The addition of anti-oxidants is well known method to improve viability and motility during cryopreservation of equine sperm cells (Ball *et al.*, 2001). Ascorbic acid (Vitamin C) is a non-enzymatic antioxidant that plays an important role in scavenging free radicals which otherwise may cause lipid peroxidation of sperm plasma membranes (Baumber *et al.*, 2000).

1.1 JUSTIFICATION

It has been shown that egg yolks of avian species other than the chicken, might be more beneficial for freezing of semen from stallion (Clulow *et al.*, 2007; Burriss and Webb, 2009), donkey (Trimeche *et al.*, 1997), bull (Su *et al.*, 2008) and ram (Kulaksizet *al.*, 2010). Also, addition of various antioxidants at different concentrations in extenders showed beneficial effect on the quality of human semen (Lewis *et al.*, 1997), equine semen (Ball *et al.*, 2001) bull semen (Beconi *et al.*, 1993) and ram semen (Azawi and Hussein, 2013) during freezing-thawing process is at different storage periods. However, there are limited reports on comparing the effect of diluents made from different avian species egg yolk and Vitamin C inclusion on semen quality of chilled (liquid) bull semen. Therefore, the current study was designed to compare the effects of egg yolk from chicken, turkey, quails and guinea fowl eggs in diluents and inclusion levels of Vitamin C (Ascorbic Acid) on spermatozoa quality of chilled bull semen stored at different periods.

1.2 OBJECTIVES OF THE STUDY

The broad objective of this study was to evaluate the effects of different avian egg yolks and Vitamin C inclusion on the characteristics of chilled bull semen.

The specific objectives were to evaluate;

1. The effect of including egg yolks from different avian species egg yolk (chicken, turkey, guinea fowl and quails) in tri-sodium citrate diluents (extenders) on characteristics of chilled bull semen.
2. The effect of including different levels of Vitamin C (0, 3 and 6mg/ml) on characteristics of chilled bull semen.

1.3 HYPOTHESIS

Experiment 1: Effect of four different avian egg yolks tri- sodium citrate extender on bovine semen characteristics.

H₀(Null Hypothesis):

Inclusion of egg yolks from different avian species in tris-based diluents have the same effect on the characteristics of chilled bull semen stored at different periods.

H_A (Alternative Hypothesis):

Inclusion of egg yolks from different avian species in tris-based diluents have different effect on the characteristics of chilled bull semen stored at different periods.

Experiment 2: Effect of Vitamin C inclusion levels in chicken and quail egg yolk tri- sodium citrate extenders on bovine characteristics.

H₀ (Null Hypothesis):

Inclusion levels of Vitamin C in egg yolk tris-based diluent have no effect on the characteristics of chilled bull semen stored at different periods.

H_A (Alternative Hypothesis):

Inclusion levels of Vitamin C in egg yolk tris-based diluents have effect on the characteristics of chilled bull semen stored at different periods.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY AND DISTRIBUTION OF CATTLE IN NIGERIA.

Over 800 breeds of cattle are recognised worldwide, some of which are adapted to the local environment, were bred by humans for special purposes (Gates, 1992). There are two main types of cattle worldwide namely *Bos indicus* cattle also known as Zebu and *Bostaurus* cattle also known as the Taurines (Felius, 1995). However, some parts of the world have other types of species existing either wild or domesticated with most of them closely related to either Taurus and Indicus or breeds that come to exist through interspecies crossing such as: the Dwarf Lulu cattle of the mountains of Nepal, the Holstein Friesian breed of Holland and the Selembu breed of India (Kohler-Rollefson *et al.*, 2009). Cattle as a species have been subjected to human interference since the advent of livestock rearing at least 8,000 years ago and the development of domesticated cattle has mirrored the evolution of human societies (Blench, 1999). Nigeria had a mean cattle population of some 13.9 million in 1990, of which 11.5 million were kept in pastoral systems and 2.4 million in villages (FAO, 2011). These were predominantly Zebu, but included 115,000 Muturu, some Keteku, N'dama and Kuri. Country-wide, the mean density of cattle is approximately 15/km², or 6.6 hectare/head (Gates, 1992). Cattle numbers increase steadily with declining rainfall, so that much of the south has low cattle densities while most of the population is in the north (Blench, 1999; Wint and Robinson, 2007).

Breeds of livestock are usually regarded as resulting from human-mediated genetic manipulation through selective breeding and reproductive isolation. Lunde and Lindtjorn (2013), evoked this process to illustrate the capacity of selection to alter the

biological composition of a species and hence support his theory of evolution by natural selection (Blench, 1999).

2.2 BREEDS OF CATTLE IN NIGERIA

2.2.1 Zebu types

Zebu is a species of cattle that is a native to the jungles of south Asia and it is the only cattle species that can easily adapt to life in the hot tropics (Lunde and Lindtjorn, 2013). The Zebu is also known as the humped cattle because it has a very distinctive hump on its back, located behind the head and neck. It was transported to Africa around the 17th century (Gates, 1992). It is known as the smallest species of cattle with height of just over a meter and this manageable size of theirs makes it easy for them to cope with the harsh tropical climate (Blench *et al.*, 1998). It has a flap of skin below its lower jaw known as a dewlap with long drooping ears. It is farmed for meat in some areas, lighter agricultural work and considered to be holy in India where it originated from (Kohler-Rollefson *et al.*, 2009). Some species of Zebu are described below.

2.2.1.1 Bunaji/White Fulani

Bunaji or White Fulani cattle is a white, black-eared and medium-horned breed, and is the most numerous and widespread of all Nigerian cattle breeds (Blench, 1999). FOA 2011 estimated that they represent some 37% of the national herd. They are found from Lagos to Sokoto, Katsina and Kano States and spread across the Nigerian Middle Belt (Hall, 1991). The only areas from which they are significantly absent are Borno, where Rahaji and Wadara predominate, and in the south-east, where there are no resident Zebu (Blench, 1999; Rege, 1999). Pastoralists generally agree that the Bunaji are superior to all other breeds of zebu in resisting disease (Rege and Tawah *et al.*, 1999).

2.2.1.2 Sokoto Gudali (Bokoloji)

There are two quite distinct types of Gudali in Nigeria, the Sokoto Gudali (Bokoloji) and the Adamawa Gudali. The Sokoto Gudali has a uniform cream, light grey or dun coat colour, the dewlap and skin folds are highly developed and the horns almost absent (Payne and Hodges, 1997). Although the Sokoto Gudali stereotypically occurs mainly in the Northwest of Nigeria, in reality it is now distributed widely throughout the country (Blench, 1999).

2.2.1.3 Rahaji

The Rahaji is one of the largest Zebu breeds and is distinguished by its deep burgundy-coloured coat, pendulous ears and long, thick horns (Blench, 1999). It is the third most numerous breed of cattle in Nigeria accounting for 22% of the National herd (FAO, 2011). The Rahaji is adapted to arid and semi-arid regions and rarely goes further south than Kaduna in the wet season, except for the isolated population on the Mambila Plateau in the south-east (Ngere, 1990). Pastoralists consider the Rahaji an extremely prestigious breed and many herds of 'white' cattle include a few Rahaji for crossbreeding (Hoffman, 2010). Nonetheless, it tolerates neither humidity-related diseases nor poor nutrition (Lunde and Lindtjorn, 2013).

2.2.1.4 Wadara

Wadara cattle are medium-sized, lightly built cattle, and are usually dark red, black, pied or brown, shorthorned and have a small erect hump (Blench, 1999) and representing about 6.6% of the Nigerian national herd (FOA, 2011). Wadara cattle are the 'indigenous' cattle of Borno region. They are frequently called 'Shuwa' in the literature, after the Shuwa Arabs who also herd them (Blench, 1993). They are said to be related breed to a white coat, the Ambala, often traded into Nigeria from Chad (Hall, 1991).

2.2.1.5 Adamawa Gudali

The Adamawa Gudali resembles the Bunaji in conformation. It is medium to large sized, with medium-length horns, and usually pied, or with a white, black, red or brown coat. It has thick, crescent-shaped horns, a pendulous hump, and a short head and muzzle (Gates, 1992; Adebamabo *et al.*, 1998). The pendulous hump is the feature that most reliably distinguishes it from the Bunaji. FAO in 2011 stated that Adamawa Gudali represent about 2% of the national herd. The Adamawa Gudali, as its name implies, is restricted to Adamawa region (Rege, 1999). The animals are favoured for ploughing, but when they become too large to pull a plough effectively they are further fattened in the compound and sent to market (Rege and Tawah, 1999).

2.2.1.5 Azawak

The Azawak is said to be native to the Azawak Valley north-east of Nigeria and is distributed along its north-western border (Felius, 1995). It is lightly built with medium-length horns. Although Azawak in Niger are commonly described as red, the Azawak that enter Nigeria are usually a light fawn colour, though they can also be white, brown, pied and black (Blench, 1999). FAO in 2011 estimated that they represent just 0.7% of the national herd. A small population of Azawak cattle exists in Nigeria throughout the year, but the majority are seasonal transhumants. Azawak are generally only found on the border north and west of Sokoto, but they could be found in the north-west of Borgu or dotted along the frontier from Sokoto to Katsina (Lunde and Lindtjorn, 2013). Azawak can trek long distances as Rahaji, they have placid temperament and can be herded away by thieves at night (Ngere, 1990).

2.2.2 Taurines

This covers animals usually described as ‘trypanotolerant’, the West African dwarf shorthorn or Muturu and the various types of Keteku, Zebu×Muturu and Zebu×N’dama crosses (ILCA, 1991; Gates, 1992). They are highly disease resistant, mainly adapted to cooler environment.

2.2.2.1 West African Dwarf Shorthorn or Muturu

The West African dwarf shorthorn or Muturu is small bodied, and blocky in conformation with short, fine-boned limbs. It has a compact body, no hump, a straight back, and a broad head. The face is slightly dished, and the horns are very short (Blench, 1993). In south-central Nigeria, the Muturu is generally black, or black and white. Animals on the Jos Plateau itself are usually black and white but are distinctly larger than lowland animals (Payne and Hodges, 1997). There are more variations in the northern populations; brown, red or tawny animals were recorded (Blench, 1999).

2.2.2.2 N’dama

N’dama cattle are native to Sene-Gambia region and adjacent parts in the west of West Africa (Blench *et al.*, 1998). They were first brought in to Nigeria from Guinea in 1939 on an experimental basis, because they were trypanotolerant and yet were larger than muturu (Blench, 1999). The N’dama has a medium-sized compact body with lyre-shaped black-tipped horns and no hump. There is a small dewlap in the male, but a fairly large head. Although those imported into Nigeria are generally light brown, there are black and pied animals in Guinea (Payne and Hodges, 1997).

2.2.2.3 Borgu Keteku

The Borgu Keteku also called Katak, Ketari, Borgu, Borgawa or Kaiama, is a trypanotolerant, stabilised Muturu x Zebu cross (Blench, 1999). It combines muturu and

Bunaji features with white, grey and black types predominating, and more occasionally red and brown. The horns are long compared with a muturu, but the hump smaller, and the legs shorter than Bunaji (Payne and Hodges, 1997). In Nigeria, Keteku herds are restricted to a narrow band along the Benin Republic border in the region usually known as 'Borgu' (Blench, 1999).

2.2.2.4 Kuri

The Kuri is a large-bodied humpless longhorn whose exact historical origin is unknown (Meghen *et al.*, 1999). The Kuri has distinctive, inflated, spongy horns unknown in any other breed. With a mean height of 1.5 m, and weight of some 550 kg, is one of the largest breeds of African cattle (Blench, 1993). Kuri are noted for their extremely variable colours and their ability to thrive in semiaquatic conditions. The nucleus of the Kuri cattle population is within the region of the Lake Chad, and along its eastern shores. In Nigeria, Kuri are found not only on the Lake but on its shores and along the Yobe valley, as far west as Gashagar (Rege, 1999). There is also a restricted export of Kuri as traction animals to the region north-east of Kano (Gates, 1992).

2.2.2.5 Crosses between local and exotic breeds

In trying to exploit the vast genetic advantages of cattle, Nigeria, years back imported the Holstein-Friesian (HF) bulls for crossbreeding with the local dairy breeds, especially Bunaji heifers and this brought about a great improvement in milk yield and reproductive potentials (Mbap and Ngere, 1995). The technology of crossbreeding has been exploited as an efficient tool of blending the adaptability of tropical cattle with the high milking potentials of exotic breeds for increased milk production (Oni *et al.*, 2001; Akpa *et al.*, 2006). Akpa *et al.*, (2011) reported the lowest percentage of calving difficulty (17%) with zero mortality in Friesian × Bunaji crosses, showing that the two breeds are highly compatible

2.3 FACTORS AFFECTING CATTLE DISTRIBUTION IN NIGERIA

2.3.1 Ecology and Feed availability

There are feed preferences ascribed to some breeds which makes them appropriate for some environments. Two or more breeds can exploit the same ecozone by making different use of feed resources (Blench, 1999). However, as the vegetation changes due to both climatic and anthropic factors, producers adapt either the breeds they use or bring in new ones. Sokoto Gudali can digest woody vegetation a distinct characteristic of the arid zone of Sokoto region that other breeds find extremely unpalatable, while the Rahaji which is the most discriminating feeder among the Zebu breeds are known to do well only in the pasture grasses of semi-arid regions (Seo and Mendelsohn, 2006). The Kuri cattle on Lake Chad, two notable features relate them to their habitat which is tolerance to insect bites and their preference for fresh grasses.

2.3.2 Diseases

Disease factors, and in particular trypanosomiasis, are usually considered to be a major factor in determining cattle distribution. The presence of disease-carrying tsetse in Nigeria has greatly affected the distribution of domestic cattle (Terblanche *et al.*, 2008). Zebu cattle cannot live for long periods in the Guinea morsitans and Guinea palpalis belts and at all in the southern part of the country except on cattle ranches (Lunde and Lindtjorn, 2013). Thousands of herds have to migrate southward during each dry season and return to the north in the wet season to get away from the dispersing tsetse. Another aspect of disease with an important impact on Muturu populations in Northern Nigeria is interaction with zebu herds (Blench, 1999). Muturu populations which have traditionally been isolated in remoter areas have had little contact with other cattle. As a

result, they are often not resistant to endemic diseases, or are more seriously affected than Zebu, which traverse a wide variety of ecological zones (Blench, 1999).

2.3.3 Animal traction

Animal traction, especially ploughing and carting, now forms a major element in household production systems in most parts of the semi-arid zone. As farmers depend increasingly on traction animals they gradually develop more discriminating preferences for particular breeds (MacDonald *et al.*, 1999). In some cases they are willing to buy animals and transport them long distances to meet their requirements. Sokoto Gudali are usually reckoned to be poor animals for ploughing because of their uncertain temperament. Similarly, Borgu Keteku, that is Muturu x Zebu crosses, are brought from Borgu as plough animals in some parts of southern Sokoto. Farmers in Adamawa tend to use the local breed, Adamawa Gudali, but this animal has barely spread outside the region. This process clearly functions to redistribute certain breeds outside their usual area (Blench, 1999).

2.3.4 Cultural and productivity preferences

Productivity parameters and cultural preferences have greatly determined the distribution of cattle in Nigeria for example, Rahaji cattle are generally considered to be the most prestigious type of Zebu among most Fulani clans. It is claimed that they are larger, give more milk and are more docile or simply more 'beautiful' (Blench, 1999). It is common for herders to introduce Rahaji bulls into more southerly 'white' herds to gradually adapt the breed to the higher rainfall zones. The Kanuri-speaking pastoralists of North-Eastern Nigeria identify strongly with the Wadara breed which they inherited 'from their forefathers' and they continue to herd it today. However, the Fulani

pastoralists who exploit the same grazing in Borno usually prefer Rahaji or Sokoto Gudali and consider the Wadara small and unprestigious (Wint and Robinson, 2007).

2.4 PHYSIOLOGY OF THE BULL REPRODUCTIVE TRACT.

The organs and glands of the male reproductive tract manufacture the male gamete (spermatozoa) and deliver it to the female reproductive tract (Karolina, 2012). Embryologically, the reproductive system is closely related to the urinary system, developing the tubules and ducts of both interdependently (Hafez, 2000). In the male adult, the urethra is a passage common to the urinary system and the reproductive tract. The male reproductive system of mammals consists of two testes (testicles) in the scrotum, accessory organs including ducts and glands, and the penis. The testes produce spermatozoa (also called sperm) and testosterone (the male sex hormone) (Strzezka, 2007). The scrotum provides a favourable environment for the production and maturation of spermatozoa. The remaining structures assist the spermatozoa in reaching their ultimate goal (the ovum of the female) in a condition conducive for fertilization of the ovum (Reece, 2005). These structures include the epididymis and ductus deferens, accessory sex glands (ampullary glands, vesicular glands, prostate, and bulbourethral glands), the urethra, and the penis (Strzezka, 2007).

2.5 SPERMATOGENESIS

Spermatogenesis is the term for all processes involved in the formation of mature male gametes from the most undifferentiated germ cells. It includes several mitotic cell divisions followed by two meiotic cell divisions, during which the chromosome number is reduced from diploid to haploid (Bearden and Fuquay, 2000). This series of cell divisions is termed spermatocytogenesis (Rowen *et al.*, 2011). Some of the cells resulting from the mitotic cell divisions of the most undifferentiated germ cells remain at

the base of the epithelium to maintain the supply of stem cells (Wierzbowski and Zukowski, 2007). Others begin the sequence of cell divisions (mitotic followed by meiotic divisions) and developmental changes to become spermatozoa. The mitotic cell divisions double the number of cells at each step, so a single spermatogonium gives rise to many spermatozoa. Meiosis entails two cell divisions and occurs only during the development of gametes in the testis and ovary (Hafez, 2000). Prior to the first division, the DNA is replicated in a manner similar to that of mitotic cell division (Rauch, 2013). This replication results in chromosomes that consist of two identical chromatids. In preparation for the first meiotic division, homologous chromosomes pair up along the middle of the cell. These homologous chromosomes are the similar chromosomes of a typical pair, each of which was contributed by one of the parent animal (Strzezka, 2007). During the first meiotic division, one chromosome of each homologous pair moves into each daughter cell (Noakes *et al.*, 2009). Individual chromosome of the homologous pairs moves to which daughter cell appears to be random. This mixing among homologous pairs provides for genetic variation among the offspring (Rowen *et al.*, 2011). After the first division, each daughter cell has a haploid number of chromosomes, but each chromosome consists of two chromatids. During the second meiotic division of the two daughter cells, each of the resulting four cells receives one of the chromatids (Reece, 2005). The overall result of meiosis is the production of four daughter cells, each of which has a haploid number of chromosomes. Spermatid is the term for the cells resulting from the second meiotic division in the seminiferous tubules. Spermatids undergo a series of functional and structural changes to become spermatozoa, and this process is termed spermiogenesis (Strzezka, 2007).

2.6 SPERM PLASMA MEMBRANE AND COMPOSITION

A sperm is entirely covered by a plasma membrane, identical to somatic cells. The sperm can be divided in several membrane domains and subdomains, depending on the function (Gadella *et al.*, 2001). The domains of the sperm head include the acrosomal and the postacrosomal region (Eddy, 2006). The plasma membrane of the acrosomal region can be divided into an acrosomal cap and an equatorial subdomain. The latter is separated by the posterior ring from the neck region of the midpiece (Eddy, 2006). Besides the specialization in function of the different domains of the plasma membrane, the lipids and proteins of the plasma membrane vary between different parts of the sperm (Gadella *et al.*, 2001).

The sperm plasma membrane consists of a phospholipid bilayer with cholesterol, complex carbohydrates and proteins, typical for plasma membranes (Meyers, 2006). The carbohydrate structures are bound to proteins or specific lipids on the outside of the plasma membrane (glycocalyx) (Gadella *et al.*, 2001). The phospholipids in the sperm plasma membrane vary between mammalian species but generally include phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin, lysophosphatidylcholine and cardiolipin (He *et al.*, 2001). In contrast to other species, sperm from bulls have a high ratio of PC to PE (Engelman, 2005). The proteins constitute about 50% of the total membrane weight and can be either peripheral to or integrated in the plasma membrane (Meyers, 2006). The cell membrane is described as a mosaic of different degrees of localized fluid areas that are also called lipid domains (Flesch and Gadella, 2000). These domains consist of certain lipids (mainly phospholipids and sterols) with certain functions. The lipids and proteins are mobile and are able to move laterally in the plane of the membrane (Silva, 2006). At room temperature, the membrane lipids are generally in a fluid (liquid

crystalline) phase, but some domains contain lipids in the gel-phase (Silva, 2006). In the gel-phase, the lipids are more clustered and less mobile (Rauch, 2013). Several reports claimed that the proteins in the plasma membrane are predominant, cause less fluidity and therefore less lateral diffusion of the lipids (Engelman, 2005). Further, the degree of fluidity depends on the type and amount of the present lipids. For instance, long chain polyunsaturated fatty acids in the phospholipids as well as a smaller amount of cholesterol increase the fluidity of the membrane at room temperature (Maldjian *et al.*, 2005)

Concurrent with the acrosomal membrane, the sperm plasma membrane has an impact on the sperm shape and volume, motility, energy production, permeability, capacitation and acrosome reaction and interaction with the oocyte (He *et al.*, 2001). The sperm membrane changes its lipid composition and location of the lipid domains during the physiological events before fertilization occurs (Rauch, 2013). Function of sperm plasma membrane under the hypoosmotic conditions, distribution and concentration of ions, or function of different organelles seem to correlate with the degree of the viability of spermatozoa after cryopreservation (Singh *et al.*, 2012).

2.7 Semen Evaluation

Semen evaluations are the several procedures carried out to find out the quality of semen after collection. Artificial insemination (AI) is the first generation reproductive biotechnological technique that has made great contribution to the genetic improvement, particularly in cattle. This profound impact would not have been possible without successful cryopreservation of semen (Gordon, 2004). A fertile ejaculate must meet certain semen parameter quality standards, such as: progressive motility, normal morphology, active energy metabolism, structural integrity and functionality of the

membrane, penetration capacity and optimum transfer of genetic material (Singh *et al.*, 2012). The main aim of semen assessment is to find one or more parameters to use to predict the fertilising ability of the semen samples. Different methods have been used for semen evaluation over the past years, but only few of these methods have been adopted for practical use in animal physiology and breeding (Said *et al.*, 2005). Most of these studies have used light microscopic evaluations of classical sperm parameters, including sperm concentration, motility, morphology, pH, colour and viability. The ideal semen analysis would be simple and effective, allowing the breeding capacity of a particular ejaculate to be predicted (Saacke *et al.*, 2000).

2.7.1 Sperm motility

Motility is used to describe the ability of sperm to move properly through the female reproductive tract (internal fertilization) or through water (external fertilization) to reach the egg. Motility is a very good parameter used to evaluate the quality of semen during breeding and cryopreservation (Saacke *et al.*, 1994). During evaluation in the laboratory semen is examined either under a bright field microscope or a phase contrast microscope using a heated stage at 35 - 39°C (Karolina, 2012). Slides and coverslips are pre-warmed on a slide warmer prior to adding warm semen (Singh *et al.*, 2012). Several fields of view are examined and the percentage of motile cells estimated to the nearest 5 or 10%. Motility is classified into two; Gross Motility- gross swirling pattern of semen which looks like a large school of fish and individual motility which is an estimate of individual sperm movement (Rekwot *et al.*, 1997). Studies have shown that in some mammals, sperm motility is suppressed by pH and calcium ion, it is suppressed by low pH in the epididymis (Saacke *et al.*, 2000). The tail of the sperm (the flagellum) confers motility upon the sperm. It has been recommended that bull semen samples kept

for processing should have greater than 60% motile sperm cell which is an indication for faster fertilization (Saacke *et al.*, 1994).

2.7.2 Sperm concentration

The sperm concentration of a semen sample refers to the number of spermatozoa per ml of the semen. This is a parameter used to evaluate the quality and fertility of semen of animals as the higher the concentration of sperm cells in the semen the higher the chances of fertilization (Saacke *et al.*, 1994; Singh *et al.*, 2012). Concentration suitable for use in breeding or cryopreservation ranges from $8-20 \times 10^6/\text{ml}$ in bulls, $2000-3000 \times 10^6/\text{ml}$ in rams, $200-300 \times 10^6/\text{ml}$ in boar and $200-500 \times 10^6/\text{ml}$ in the stallion (Sorensen Jr, 1997). Concentration is usually determined using specialized equipment containing a spectrophotometer called the haemocytometer (Rekwot *et al.*, 1987; Sorensen Jr, 1997). The concentration of sperm in the neat semen is needed to determine how to dilute semen and provide adequate sperm in each breeding dose (Vishwanath, 2003). The number of sperm ejaculated varies greatly among males but is dependent on age, size of the testes, and the efficiency of spermatogenesis/gram of tissue (Strzezka, 2007). Sperm production on any day is also dependent on the collection frequency and the intensity of the sexual stimulation employed (Karolina, 2012).

2.7.3 Sperm viability

The viability of sperm cells in a semen sample is a strong parameter used to determine the quality as well as fertility of the semen (Singh *et al.*, 2012). Sperm viability is the percentage of sperm cells in the semen that are alive (Karolina, 2012). The plasma membrane of the sperm helps to detect this parameter. The staining procedure using histological stains such as eosin-nigrosin that would only penetrate the sperm with a

compromised plasma membrane (Vishwanath, 2008). Sperm smear were then dried on a slide and counted as viable (unstained) or dead (stained) (Butswat *et al.*, 2001). Other procedures use a combination of fluorescent dyes such as sybr-14 which stains viable sperm green and propidium iodide which stains dead sperms red (Vishwanath, 2008). Semen samples are expected to have not less than 70% viable sperm by vital stain assay (Saacke *et al.*, 1994).

2.7.4 Semen colour and consistency

It has been known that bull semen which is concentrated has creamy, milky or creamy white and opaque colour (Hafez, 2000; Bearden and Fuquay, 2000). On the other hand, semen with few spermatozoa and that from animal with reproductive system infections has been reported respectively to be translucent and curdy in appearance (Hafez, 2000). Some bulls have been known to consistently produce yellow semen which is due to a harmless pigment known as riboflavin. However, the semen should be free from hair, dirt, urine, blood, faeces and other contaminants (Hafez, 2000; Bearden and Fuquay, 2000). Semen with the spermatozoa concentration of one million to 1.2 million per cubic millimeter or higher has been classified as creamy consistency while the spermatozoa with concentration of 5×10^5 to 6×10^5 per cubic millimeter has been classified as a thin milky consistency, and those with the spermatozoa concentration of less than 3×10^5 spermatozoa per cubic millimeter has been classified as watery, translucent or clear (Bearden and Fuquay, 2000).

2.7.5 Semen Volume

The volume of the ejaculate is readily measured by collecting the sample directly into a graduated vial (Bearden and Fuquay, 2000), or by weighing the tubes after semen collection on top-loading balance, and later converting the reading into milliliter by

using a computer program. The latter has been known to reduce error associated with visual reading of the tube especially when small volume or bubbles are found by (Bearden and Fuquay, 2000). The volume has been reported to decline when young bulls are used or when there is frequent ejaculation or incomplete or failure of ejaculation and in bilateral seminal vesiculitis (Hafez, 2000). In summary a number of factors like season of the year, method of collection, and the sexual preparation of the bull have been known to affect semen volume (Singh, 2012). The normal volume of semen of the bull has been known to be within the range of 5 to 8 ml (Hafez, 2000) and 4 to 8 ml, 0.8- 1.2ml in rams, 150-200ml in Boar and 60-100ml in stallions per ejaculate (Sorenson Jr, 1997). Nevertheless, small volume, unless accompanied by a low semen concentration has also been reported to be harmless (Hafez, 2000).

2.7.6 Semen pH

The pH of semen has been reported to be measured by pH paper, bromothymole blue or a pH meter (Vithwanath, 2008). The pH of bull semen is slightly acidic, about 6.7, and has been reported to rise when there is incomplete ejaculation, excessive use of the bull or in yearling bulls and in pathologic situation of the testis, epididymis or ampulla. Dense semen samples which possess excellent motility have been known to show lower values of pH (Foote, 2002). It has been shown that pH of 6 – 7 are suitable for liquid storage of bovine semen with good motility scores (Foote *et al.*, 2002).

2.7.7 Sperm morphology

It is known that abnormalities in the sperm cell can bring about decreased ability to reach the site of fertilization, inability to fertilize oocytes, or inability to sustain embryonic development (Gordon, 2004). The sperm cell is a highly structured cell that is fashioned to deliver DNA to the oocyte (Meyers, 2006; Gravance *et al.*, 2009). To

achieve this goal, the DNA is highly condensed and packaged on the nuclear matrix in a unique and specific species manner. Alterations in the packaging or DNA content of sperm results in changes in the morphology of the sperm head. The tail of the sperm contains the locomotion apparatus and without its proper function cannot deliver the DNA in the sperm head to the oocyte (Gravance *et al.*, 2009).

Semen from males contains some abnormally formed spermatozoa, which are not associated with lower fertility rates until the proportion of abnormal sperm exceeds about 20% and even then certain types of abnormalities may not be associated with infertility (Singh *et al.*, 2012). At least 200 sperm cells should be counted and skill is required in classifying the cell types (Chenoweth, 2005). Morphological abnormalities of spermatozoa can be primary (failure of spermatogenesis), secondary (occurs during the passage of spermatozoa through the epididymis) or tertiary (damage resulting from improper handling of semen). Some of these abnormalities observed include coiled tails, bent tails, tapered heads, midpiece droplet, detached head, double heads micro heads, macro heads and so forth (Rekwot *et al.*, 1987; Singh *et al.*, 2012) Hence, ejaculates should have approximately greater than 70% normal sperm cells and not more than 20% primary abnormalities (Meyers, 2006).

2.8 FACTORS AFFECTING SEMEN QUALITY

2.8.1 Age

Environmental factors such as age and genotype has effect in sperm production and semen quality of bulls. There is an increase in sperm minor defects and decrease in sperm motility with an increase in bulls age (Brito *et al.*, 2002). Bulls which are two years or younger have been known to have higher percentage of head abnormality, on the other hand bulls which are older than seven years has not been recommended to be

used for natural mating (Foote *et al.*, 2002). Such bulls have reduced sperm production owing to higher incidence of lesions such as testicular senile fibrosis and tubular calcification, which decrease their fertility (Foote, 2002; Hunderra, 2004). Similarly it has been reported that testicular size, scrotal circumference and body weight are positively correlated with age and semen volume, quality and amount of mature spermatozoa have also been found to be positively correlated with testicular size and scrotal circumference (Hunderra, 2004; Blezing;er, 1999). If every parameter has been kept equal, best semen quality has been obtained from bulls which are a little underweight (Fuerst-Waltl *et al.*, 2006).

2.8.2 Breeds

Libido or sex drive and mating ability in the bull has been reported to be influenced by genetic factors, and lack of sexual desire has been found more common in some strains and breeds of cattle (beef breeds and *Bos indicus*) than others (Hafez, 2000, Hunderra, 2004).Some European breeds, such as Polled Herefords have been known to give good quality semen during winter and early spring when the weather is cool; Brahman types, bulls have been reported to have better semen quality during warmer months (Terblanche *et al.*, 2008). *Bos Indicus* bulls have been reported to have a significantly higher sperm concentration and higher sperm morphologic defects than *Bos Taurus* (Brito *et al.*, 2002; Nichi *et al.*, 2006).But whether such difference is due to breed or seasonal difference needs investigation.

2.8.3 Nutrition

Nutritional deficiencies, in the bull have been reported to delay the onset of puberty and depress production and characteristics of semen (Violeta *et al.*, 2010). The negative effect of under feeding has been found to be more serious in young than adult bulls

(Hafez, 2000). Underfeeding, protein malnutrition and certain low vitamins interfere with the regulation of hypothalamo-pituitary axis thereby altering gonadalsteroid production. Also responses of accessory glands to testosterone has been found to be reduced (Hunderra,2004). On the other hand, obesity and overfeeding have been known to reduce sexual function in a bull (Blezinger, 1999; Hafez, 2000). These have been associated with excessive fat deposition in the scrotum which may interfere with testicular thermoregulation (Hunderra, 2004).

2.8.4Temperature

Climatic factors like ambient temperature; humidity, convection current, and solar radiation affect testicular function directly or through neuro-hormonal mechanism (Taylor *et al.*, 2002). Elevated body temperature during periods of high ambient temperature or pyrexia from disease has been noted to cause testicular degeneration and reduce the percentage of normal and fertile spermatozoa in the ejaculate (Hundera, 2004; Nichi *et al.*, 2006).Body cooling either via water sprinkling or fanning has been reported to decrease percentage of dead sperm, sperm abnormality and acrosome damage in Murrha Buffalo bulls (Mandal *et al.*, 2002).

2.8.5Scrotal circumference

Scrotal circumference has been recognized to be highly heritable, and can serve as an indicator of puberty, total sperm production, semen quality, pathologic condition of testes, potential sub fertility or infertility of bulls (Fuerst-Waltl *et al.*, 2006). Consequently, failure to select properly for this trait has the potential of decreasing fertility and production for the generations to come (Foote, 2002). The bulls with larger testes have been known to give larger volume of semen, larger sperm motility and percentage of normal spermatozoa (Hunderra, 2004).

2.8.6 Disease of testis, epididymis and accessory glands

Pathologic condition of testis, epididymis, and seminal vesicle such as testicular degeneration, orchitis, epididymis and seminal vesiculitis respectively have shown to interfere with fertility by disturbing spermatogenesis or sperm maturation, leading to abnormal semen characteristics or preventing the passage of spermatozoa from testes to urethra (Blezinger, 1999; Hafez, 2000). According to Hafez (1993) and Huderra, (2004) seminal changes that occur during testicular degeneration include: increase in immature and abnormal sperm with normal motility, but as the disease progresses the ejaculate becomes thin and watery, due to low concentration of the spermatozoa. These two authors also reported the appearance of giant cells, azoospermia or necrospermia in severe testicular degeneration. When the bull suffers from inflammation of the testes (orchitis), some of the seminal changes expected are asthenozoospermia, oligozoospermia, teratozoospermia, giant cells, white blood cells, and red blood cells with normal semen volume (Mathevon *et al.*, 1998). Similarly, poor semen characteristics and contamination of semen with inflammatory cells have been reported as the main features observed when there is epididymitis (Jimenez-Severiano *et al.*, 2003).

2.9 HISTORY OF ARTIFICIAL INSEMINATION IN CATTLE.

Research on artificial insemination (AI) dates back to several centuries. Its commercial application in a dog, rabbits and horses was mentioned in a scientific publication (Foote, 2002). An important step in artificial insemination was the work of the Russian physiologist Ivanov, in 1912, which not only advanced artificial insemination in the dog and the pig, but also achieved pregnancy rates in horses that were comparable to natural service (Gordon, 2004). Artificial vaginas (AVs) were designed for dogs, cattle, horses

and sheep in Europe at the beginning of the 20th century (Gordon, 2004). Inspired by Ivanov, artificial insemination and related research was conducted in Japan (Foote, 2002). The first book on artificial insemination was published by a British Biologist in 1933 (Rekwot *et al.*, 1997) and three years later, the first cooperative dairy AI organization was founded in Denmark (Foote, 2002). Several inventions improved artificial insemination and helped with its worldwide application. This included: the development of the rectally guided trans cervical method of artificial insemination in cattle by Danish veterinarians in 1937 (Gordon, 2004) which allowed a reduction of the insemination dose. The observation that egg yolk in a buffer can preserve bovine semen for several days (Rauch, 2013) and the discovery of glycerol which made the cryopreservation of bovine semen possible (Gordon 2004). Also, the addition of antibiotics to the extender to minimize bacterial contamination and limit venereal diseases was observed (Rauch, 2013). Further research led to improvement and major changes in the storage of semen. Egg yolk based extender was improved to contain Tris- and citrate-buffers instead of a phosphate buffer (Rauch, 2013). Almquist and his co-workers also established an extender for bovine semen that was based on whole-milk (Zemjams, 1971). In 1960, liquid nitrogen replaced solid carbon dioxide (dry ice) thus allowing the long term storage of frozen semen in specialized containers (Moussa *et al.*, 2002). The use of straws instead of freezing the semen in glass ampules was an important modification to simplify the packing of cryopreserved semen (Gordon, 2004). Methods of detecting estrus, synchronization of estrus and timed artificial insemination made it possible to improve conception rates and therefore distribute superior genetic material worldwide (Moussa *et al.*, 2002). Advances include the production of sexed semen by flow cytometry (Moussa *et al.*, 2002).

2.10 SEMEN PRESERVATION

Preservation of bull spermatozoa over a long period of time in vitro without losing their viability forms an integral aspect in artificial insemination in cattle breeding (Chaconet *et al.*, 1999). Different methods have been adopted for preservation of mammalian spermatozoa, but those widely used for bull spermatozoa are chilled storage, deep freezing and room temperature storage methods (Dorado *et al.*, 2010; Singh *et al.*, 2012). Several factors have been reported to be responsible for low survival rate of spermatozoa during storage which include effects of type and composition of diluents (extenders), rates of cooling, dilution of semen and length of storage period (Bailey *et al.*, 2000). Semen used for cryopreservation is more rigorously evaluated than semen collected for a breeding soundness exam in all evaluation categories. Since sperm cells need to survive both the freezing and thawing process, starting with the highest quality semen is essential (Butswat, *et al.*, 2001; Singh *et al.*, 2012).

2.11 METHODS OF SEMEN PRESERVATION

2.11.1 Room temperature(Ambient Temperature) storage

The room temperature is a storage at about 20-35⁰C, a short term method of storing semen (0-4 day) (Butswat, *et al.*, 2001). Semen stored at room temperature has been reported to be of higher quality than frozen semen due to the incidence of cold shock experienced by sperm cell during freezing- thawing processes (Karolina, 2012). However, quality of semen kept at room temperature decreases with increase in storage time as a result of metabolic processes in the sperm cells which is not prevalent in samples stored at lower temperatures (Singh *et al.*, 2012).

2.11.2 Chilled(liquid) semen

Chilling is done using extenders based on either ionic or non-ionic substances that prevent changes in osmolarity and act as a buffer against changes in pH (Vithwanath and Shannon, 2000). Though chilling is found to preserve sperm cells for a relative shorter period of time when compared to the freezing method of preservation (Singh *et al.*, 2012), sperm cells are usually more qualitative with higher fertility potential (DeJarnette, 2005; Gadea *et al.*, 2005).

2.11.3 Deep Freezing

Freezing method of preserving sperm cells is commonly used in cattle breeding industries due to its ability to store cells for longer period of time when compared to the other two methods (Vishwanath and Shannon, 2000). However, it has been observed that the process induces extensive biophysical and biochemical changes in the membrane of the cells (Watson, 2000), thereby decreasing the fertility potential of the cells (Dejarnette, 2005; Gravance *et al.*, 2009).

Freezing method of semen preservation has been found to preserve semen for longer period of time compared the to other two methods of preservation, however, due to cold shock during preservation (Karolina, 2012), sperm cells record more abnormalities when examined before insemination and it has lower percentage of viable sperm cells compared to cells preserved using the chilling method and hence lower fertility (Chenoweth, 2005).

2.12 SEMEN EXTENDERS (DILUENTS)

The choice of semen diluent is crucial to the viability of the spermatozoa during storage; the sperm are basically confronted with two major challenges namely, cold and

warming shocks, and the formation and dissolution of ice which results in changes in osmolarity and damage due to ice crystals (Dorado *et al.*, 2010; Rauch, 2013). Without specific freezing and thawing rates and diluents, the sperm would not survive the cryopreservation process. Extenders for storing cooled or cryopreserved semen have to be based on ionic or non-ionic substances that prevent changes in osmolarity and act as a buffer against changes in pH (Vishwanath and Shannon, 2000). In addition, penetrating cryoprotectants like glycerol or dimethyl sulfoxide (DMSO) and non-penetrating cryoprotectants, like glucose or fructose (Bergeron *et al.*, 2007), reduce the intracellular ice-crystal formation and serve concurrently as energy sources for the spermatozoa. Antibiotics are generally added to minimize the growth of microorganisms originating from the seminal plasma or by contamination (Vishwanath and Shannon, 2000). Furthermore, cold shock has to be antagonized and can be accomplished by a source of lipoproteins or high molecular weight material such as egg yolk, milk or plant lipids. Heated milk proved to be an appropriate diluent for cooled and frozen bovine semen (Bergeron *et al.*, 2007). Freezing extenders based on milk are prepared with 10% whole milk or skim milk, 7% glycerol and antibiotics (Vishwanath and Shannon, 2000). The addition of lactose to the extender enhances the cryoprotective effect of milk (Thieber and Guerin, 2000). The casein micelles in the milk are believed to be responsible for the sperm protection during cryopreservation (Yamauchi *et al.*, 2009). Plant-based extenders for semen are commercially available and are based on a soybean lecithin and the protective mechanism is likely based on the binding of the lipid (soybean lecithin) to the sperm membrane (Ricker *et al.*, 2006). Extenders that are free of components of animal origin have a lower risk of microbial contamination (Aires *et al.*, 2003). Contaminated extenders could spread diseases or even introduce exotic diseases (Van Wagendonk-deet *et al.*, 2000) respectively.

Furthermore, bacterial contamination can cause production of endotoxins that are harmful to the sperm directly (Thieber and Guerin, 2000). Compared to egg yolk extenders, the laboratory quality measures of plant-based extenders are similar (Aires *et al.*, 2003). However, the field fertility is lowered for soy bean extract extenders when several fertility parameters are considered in a multiphasic model (Van Wagendonk *et al.*, 2000).

2.13 SEMEN COOLING AND COOLING RATES

Bull spermatozoa behave like other tissues in their response to temperature changes. There is an irreversible loss of viability as a result of sudden fall in temperature referred to as 'cold shock' (Graham and Moce, 2005). Associated to this phenomenon is a reduction in the rate of glycolysis and dehydrogenase activity which may be responsible for loss in motility of the spermatozoa. Although egg yolk and other cryoprotectants are said to reduce the effect of cold shock but cooling in a fast manner can be a disadvantage (Bailey *et al.*, 2000). The widely adopted way is by placing the ejaculate in test tubes and placing them in water baths that's set between 30-37°C before transferring test tubes to a refrigerator in the case of freezing or bijour bottles then in a flask before placing in the refrigerator during chilling storage (Dorado *et al.*, 2010).

2.14 SEMEN DILUTION AND DILUTION RATES

The extent to which semen can be diluted during semen preservation before carrying out artificial insemination is critical to the viability of the spermatozoa (Dorado *et al.*, 2010). Various temperatures at which dilution can be carried out have been adapted and there seems to be three approaches namely; dilution at body temperature (30-37°C), room temperature(14-25°C) and at 5°C which involves two major steps (DeJarnette, 2005). Semen dilution is carried out on volume to volume (semen:diluent) basis or to a

fixed sperm concentration. However, motility is best maintained at 1:10 dilution rate and worst at 1:1 in bull semen (Graham and Moce, 2005). Semen with cell concentration not less than 50×10^6 spermatozoa/ml was shown to be acceptable during dilution (Bailey *et al.*, 2000).

2.15 LENGTH OF STORAGE

Under normal storage conditions (room temperature) semen cannot last longer than two (2) days after collection (Thieber and Guerin, 2000; Dorado *et al.*, 2010). Fertility of the semen generally declines with storage although with a good diluent and lower temperature, good motility can still be maintained over a longer period of time as to when no proper diluent and higher temperatures are used (Foote, 2002).

2.16 EGG YOLK AND EGG YOLK EXTENDERS

The use of egg yolk in extenders for cooled bovine semen dates back to 1939 in cooled bovine semen (Manjunath *et al.*, 2002; Rauch, 2013). Foote, 2002 contributed to modern extender recipes contain 16%, 20% and 24% chicken egg yolk which are favourable in the freezing extender (Vishwanath, 2003; Foote, 2002). Besides using whole egg yolk for the semen extender, a fraction with fewer particles (clarified yolk) or only the low-density lipoprotein fraction of the yolk can be used to prepare an efficient semen extender (Moussa *et al.*, 2002).

2.17 COMPOSITION OF EGG YOLK

Dried chicken egg yolk consists of 63% lipids and 33% proteins. The fresh egg yolk can be fractionated into 78% plasma and 22% granules (Anton, 2006). The granules contain 16% high density lipoproteins (HDL), 4% phosphovitin and 2% low-density lipoproteins (LDL) (Manjunath *et al.*, 2002). In the yolk plasma, the main component is LDL (66%),

followed by livetins (10%) (Foote, 2002). Phosvitin is a highly phosphorylated protein with bactericidal and antioxidant properties (Anton, 2007). Livetins correspond to serum proteins and are made of albumin, α -2-glycoprotein and immunoglobulin Y (IgY) (Rauch, 2013). Low-density lipoproteins are sphere-shaped with a liquid lipid core that is made of triglycerides and cholesterol esters (Watson, 2000). This core part is surrounded by one layer of phospholipids. Apoprotein and some cholesterol are incorporated into the phospholipid layer (Kulaksiz *et al.*, 2010). It is possible to fractionate low density lipoproteins into a population with a higher and a lower density (LDL1 and LDL2, respectively) by ultracentrifugation (Rauch, 2013). High-density lipoproteins were formerly known as lipovitellin and are associated with the phosvitins to form the granules. They are composed of 75 to 80% proteins and 20 to 25% lipids of which the latter contains 65% phospholipids, 30% triglycerides, and 5% cholesterol (Foote, 2002). The phospholipids of the whole egg yolk consist of Cardiolipin, Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylserine (PS), Phosphatidylcholine (PC), and Sphingomyelin (SPH) (Foote, 2002). These phospholipids, cholesterol and low density lipoproteins in egg yolk may be the factors that provide protection to sperm against cold shock during cooling- thaw process of cryopreservation at the same time nourishing the cells (Anton *et al.*, 2006; Kulaksiz *et al.*, 2010); although, avian species and even breeds of specie have different combination of these factors (Su *et al.*, 2008; Burris and Webb, 2009).

2.18 FREE RADICALS

Free radicals are short lived reactive chemical intermediates, which contain one or more unpaired electrons (Sanocka and Kurpisz, 2004). They induce cellular damages when they pass this unpaired electron onto nearby cellular structures, resulting in oxidation of cell membrane lipids, amino acids in proteins or within nucleic acids (Agarwal *et al.*,

2004). Free radicals are also known as a necessary evil for intracellular signals involved in the normal process of cell proliferation, differentiation, and migration (Ford, 2001). In the reproductive tract, free radicals also play a dual role and can modulate various reproductive functions (Du Plessis *et al.*, 2008). Excess of free radicals generation frequently involves an error in spermiogenesis resulting in the release of spermatozoa from the germinal epithelium exhibiting abnormally high levels of cytoplasmic retention (Sanocka and Kurpisz, 2004).

During semen preservation, sperm cells are usually exposed to oxygen and visible light radiation during the process at cryopreservation leading to the formation of reactive oxidative species also known as free radicals (Aysun, 2009) such as Hydroxyl ion, super oxide, lipid peroxides, single oxygen. Excess of Reactive oxygen species (ROS) impairs motility and capacity of fertilization due to the oxidative stress damage incurred on the sperm cells by free radicals (Bucak *et al.*, 2010).

2.19 REACTIVE OXYGEN SPECIES (ROS)

One of the most important factors contributing to poor quality semen has been reported to be oxidative stress. Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen derived oxidants commonly known as ROS (Bucak *et al.*, 2010). Uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress (OS) which is harmful to spermatozoa (Desai *et al.*, 2009). All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress (Agarwal *et al.*, 2008).

ROS are formed as necessary by-products during the normal enzymatic reactions of inter- and intracellular signalling (Agarwal and Prabakaran, 2005). Mammalian spermatozoa represent a growing list of cell types that exhibit a capacity to generate

ROS when incubated under aerobic conditions, such as, hydrogen peroxide, the superoxide anion, the hydroxyl radical, and hypochlorite radical (Agarwal *et al.*, 2005). Due to their highly reactive nature, ROS can combine readily with other molecules, directly causing oxidation that can lead to structural and functional changes and result in cellular damage (Ford, 2001).

2.20 ORIGIN AND TYPES OF REACTIVE OXYGEN SPECIES (ROS)

ROS represent a broad category of molecules that include the collection of radicals (hydroxyl ion, superoxide, nitric oxide, peroxy, etc.) and nonradicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives (Sikka, 2001). Reactive nitrogen species (nitrous oxide, peroxyxynitrite, nitroxyl ion, etc.) are free nitrogen radicals and considered a subclass of ROS (Ford, 2001).

In male, two ROS generating systems are possibly involved a hypothetical NADH oxidase at the level of sperm membrane and low sperm diaphorase mitochondrial NADH-dependent oxidoreductase (Sariozkanet *et al.*, 2009). In bovine semen, ROS are generated primarily by dead spermatozoa via an aromatic amino acid oxidase catalysed reaction (Garrido *et al.*, 2004).

Leukocytes and immature spermatozoa are the two main sources of ROS. Leukocytes particularly neutrophils and macrophages have been associated with excessive ROS production and they ultimately cause sperm dysfunction (Agarwal *et al.*, 2004).

Two of the main factors contributing to Reactive Oxidative Species accumulation *in vitro* are the absence of endogenous defence mechanism and second exposure of gametes and embryos to various manipulation techniques as well as environment that can lead to generation of oxidative stress (Du Piessis *et al.*, 2008). Levels of Reactive Oxidative Species may rarely fluctuate within a fertile individual, but, do not affect

sperm concentration and motility. This may be due to the presence of adequate antioxidant defence mechanisms in the healthy individuals. The fluctuations in ROS levels might be due to transient subclinical infection and transient abnormalities in spermatogenesis such as, retention of cytoplasm or periodic presence of abnormal spermatozoa in semen (Desai *et al.*, 2009).

2.21 Positive and Negative effects of Reactive Oxidative Species

The production of ROS is a normal physiological process but an imbalance between ROS generation and scavenging activity is detrimental to the sperm and associated with male infertility (Garrido *et al.*, 2004). ROS generated by spermatozoa play an important role in normal physiological processes such as, sperm capacitation, acrosome reaction, maintenance of fertilizing ability, and stabilization of the mitochondrial capsule in the mid-piece in bovine for example single oxygen and hydrogen peroxides.(Agarwal *et al.*, 2008). Controlled generation of ROS may function as signalling molecules (second messengers) in many different cell types; and are important mediators of sperm functions. It has been shown that superoxide anion is required for the late stage of embryo development such as, two germ cell layers and egg cylinder (Ford, 2001). Although a significant negative correlation between ROS and IVF fertilization rate has been reported (Agarwal and Prabakaran, 2005), controlled generation of ROS has been shown to be essential for the development of capacitation and hyperactivation of the two processes of sperm that are necessary to ensure fertilization(Bucak *et al.*, 2008). In vivo physiological concentrations of ROS are involved in providing membrane fluidity, maintaining the fertilizing ability and acrosome reaction of sperm (Bucak *et al.*, 2010). Therefore, the maintenance of a suitable ROS level is essential for adequate sperm functionality. ROS cause adverse effects on the sperm plasma membrane, DNA, and physiological processes, thereby, affecting the quality of spermatozoa. The axosome

and associated dense fibers of the mid-piece in sperm are covered by mitochondria that generate energy from intracellular stores of ATP depletion (Bucak *et al.*,2007). Excessive ROS impairs motility and capacity of fertilization. Additionally, cold shock arising from other stress plays an important role in the moulding of membranes by determining their sol gel balance and the dynamic status that affects the fusion of the plasma membrane of the male and female gametes (Bucak *et al.*,2007). The assumption that ROS can influence male fertility has received substantial scientific support (Agarwal and Prabakaran, 2005).

Mammalian spermatozoa membranes are rich in polyunsaturated fatty acids (PUFAs) and are sensitive to oxygen-induced damage mediated by lipid peroxidation. They are sensitive to ROS attack which results in decreased sperm motility, presumably by a rapid loss of intracellular ATP. Due to this, axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects with deleterious effects on sperm capacitation and acrosome reaction occurs (Desai *et al.*, 2010).

2.22 LIPID PEROXIDATION

Lipid peroxidation (LPO) is the mechanism of ROS-induced damage to spermatozoa includes an oxidative attack on the sperm membrane lipids cascade (Ford, 2001).Mammalian spermatozoa are known to be susceptible to loss of motility in the exogenous oxidant, as a consequence of LPO (Bansal and Bilaspuri, 2008^c). The susceptibility of ruminant spermatozoa to oxidative stress is a consequence of the abundance of PUFAs in sperm plasma membrane, the presence of which gives the membranes fluidity and flexibility which help the sperm to engage in membrane fusion events associated with the fertilization. Unfortunately, the presence of double bonds in these molecules makes them vulnerable to free radicals attack and the initiation of LPO cascade (Suarez, 2008). This results in a subsequent loss in membrane and

morphological integrity, impaired cell functions, along with impaired sperm motility and induction of sperm apoptosis (Bucak *et al.*, 2010).

Lipid peroxides are spontaneously generated in the sperm plasma membrane and are released by the action of phospholipase A₂ and are capable of inducing DNA damage thus decrease in fertility during storage of semen (Bansal and Bilaspuri, 2007). The peroxides are generally associated with decreased sperm functions and viability, but, have also a significant enhancing effect on the ability of spermatozoa to bind with homologous and heterologous zona pellucida (Bucak *et al.*, 2010).

2.23 ROLE ON ANTIOXIDANTS IN CHARACTERISTICS OF SPERMATOZOA DURING STORAGE

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma to prevent oxidative damage (Bansal and Bilaspuri, 2008^d). An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility (Kumar and Mahmood, 2001). Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the oxidative stress (Bansal and Bilaspuri, 2009). Antioxidants, in general, are the compounds and their reactions usually dispose, scavenge, and suppress the formation of ROS, or oppose their actions. Manganese ion (Mn²⁺) enhances sperm motility, viability, capacitation and acrosome reaction by decreasing the oxidative stress (Bucak *et al.*, 2009). Extracellular addition of Mn²⁺ ions also enhances the level of cAMP by stimulating Calcium ions (Ca²⁺) or Magnesium ions (Mg²⁺) ATPase which leads to activation of calcium channel opening, thereby depositing more Ca²⁺. Thus, Mn²⁺ promotes the acrosome reaction (Bansal and Bilaspuri, 2008^b). Thiol groups also play an important role in detoxification and antioxidation of ROS, besides maintaining the intracellular redox status. These groups serve as defence mechanisms of sperm cells to fight against oxidative stress

(Bansal and Bilaspuri, 2008^a). A variety of biological and chemical antioxidants that attack ROS and LPO are presently under investigation (Bilaspuri and Bansal, 2008^c). Vitamin E and C (Antioxidant) may directly neutralise the effects of free radicals such as peroxy and alkoxy generated during ferrous ascorbate-induced LPO, thus it is suggested as major chain breaking antioxidant (Bansal and Bilaspuri, 2008b). Recent studies have demonstrate that supplementation of cryopreservation extenders with antioxidants has been shown to provide a cryoprotective effect on bull, ram, goat, boar, canine, and human sperm parameters (Bucak *et al.*, 2010). In goats, supplementation with antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation technique (Bucak *et al.*, 2010).

2.24 TYPES OF ANTIOXIDANTS

2.24.1 Enzymatic Antioxidants

Enzymatic antioxidants are also known as natural antioxidants; and can neutralize excess ROS and prevent them from damaging the cellular structure (Suarez, 2008). Enzymatic antioxidants are composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR) which can cause reduction of hydrogen peroxides to water and alcohol (Agarwal and Prabakaran, 2005). Catalase presence in sperm has been demonstrated for ram and cattle and it has a potential role in ageing process and control of oxidative stress in cells, mainly resulting from H₂O₂ (Bansal and Bilaspuri, 2007). GSH is also a cofactor for GSHPx that catalyses the reduction of toxic H₂O₂ and other hydroperoxides, protecting the mammalian cells from oxidative stress (Bucak *et al.*, 2007). Glutamine (5 mM) has been provided a cryoprotective effect by improving postthaw motility, membrane integrity, and catalase enzyme activity in ram semen (Uysal and Bucak, 2007). Supplementation

of inositol in the extender can improve the motility of frozen thawed bull sperm (Bucak *et al.*, 2010). Inositol has cryoprotective and anti-oxidative properties resulting in higher antioxidant GSH activity, acrosome integrity, and intact morphological rates (Bucak *et al.*, 2010). Cysteine is a low-molecular weight amino acid containing thiol; it is a precursor of intracellular glutathione (Reddy *et al.*, 2007). It has been shown to penetrate the cell membrane easily, enhancing the intracellular GSH biosynthesis both in vivo and in vitro and protecting the membrane lipids and proteins due to indirect radical scavenging properties (Pasqualatto *et al.*, 2000). It is also thought that GSH synthesis under in vitro conditions may be impaired because of deficiency of cysteine in the media, due to its high instability and auto-oxidation to cysteine (Bucak *et al.*, 2010).

2.24.2 Non-enzymatic Antioxidants

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The body's complex antioxidant system is influenced by dietary intake of antioxidants, vitamins, and minerals such as vitamin C, vitamin E, zinc, taurine, hypotaurine, and glutathione (Agarwal and Prabakaran, 2005). Glutathione is a molecule found at mM level in a number of cells, able to react with many ROS directly (Bucak *et al.*, 2007). Cysteine has cryoprotective effect on the functional integrity of axosome and mitochondria improving post-thawed sperm motility. It has been proved that thiols such as glutathione and cysteine prevented the loss of sperm motility in frozen thawed bull semen (Reddy *et al.*, 2007). Cysteine has been shown to prevent the loss in motility of frozen thawed bull, ram, and goat semen and to improve viability, the chromatin structure, and membrane integrity of boar sperm during liquid preservation (Reddy *et al.*, 2007). Cysteine has improved the porcine oocytes maturation and fertilization in-vitro (Pasqualatto *et al.*, 2000). Trehalose or taurine, a sulfonic amino acid, acts as non-

enzymatic scavenger that plays an important role in the protection of spermatozoa against ROS, in case of exposure to aerobic conditions and the freezing—thawing process (Ford, 2001). A non-permanent disaccharide has a protective action related both to osmotic effect and specific interactions with membrane phospholipids, rendering hypertonic media, causing cellular osmotic dehydration before freezing and then decreasing the amount of cell injury by its crystallization (Bansal and Bilaspuri, 2007). Trehalose performs better cryoprotection post-thaw fertilizing ability in ram, bull, and mouse sperm due to diminished death and damage of sperm (Bansal and Bilaspuri, 2007). A study has demonstrated that antioxidant capacity of trehalose is observed upon the performance of incubation at 37°C for 3 hours and no difference is obtained at 0 h post-thawing of ram semen (Bansal and Bilaspuri, 2007). Tuarine displayed antioxidative properties by elevating catalase level in close association with superoxide dismutase concentration in ram, rabbit, and bull spermatozoa (Ford, 2001). Hyaluronan, an essential component of the extracellular matrix and non-sulfated glycosaminoglycan, is involved in important physiological functions such as motility, capacitation of spermatozoa and preserve post-thaw spermatozoa viability, and in vitro membrane stability (Bansal and Bilaspuri, 2007). Hyaluronan improves sperm motility, viability and membrane integrity after freezing and thawing procedure and decrease polyspermy with declining motility in humans and boars (Bansal and Bilaspuri, 2007). Bovine serum albumin (BSA) is known to eliminate free radicals generated by oxidative stress and protect membrane integrity of sperm cells from heat shock during freezing-thawing of canine semen (Reddy *et al.* , 2007). Carotenoids such as beta-carotene and lycopene are also important components of antioxidant defence. Beta carotenes protect the plasma membrane against LPO in rat (Reddy *et al.*, 2007). Addition of antioxidants vitamin E, butylated hydroxytoluene (BHT), and Tempo to extended turkey semen

improves sperm survival and membrane integrity and reduces the loss of motility after 48 h of storage (Reddy *et al.* , 2007).

Since long it has been known that supplementation of culture media with antioxidants such as, ROS scavengers, disulfide reducing, or divalent chelators prolongs the motility of reactivated bull spermatozoa after freezing and thawing (Yousefet *al.*, 2003). It has been suggested that antioxidant therapy appears to be efficient not only in vitro but also in vivo (Yousefet *al.*, 2003). Numerous antioxidants have proven beneficial in protecting damaging effects of ROS on sperm movement and against oxidative damage (Cheema *et al.*, 2009).

2.25 CRYOPRESERVATION/ THAWING – OXIDATIVE STRESS

Semen cryopreservation is an important technology in physiology which has several advantages to the livestock industry (Uysal and Bucak, 2007). Development in semen cryopreservation techniques requires deep knowledge of the gamete physiology and the biochemical processes that takesplace during semen collection, processing, and freeze/chilling-thawing. The constant performance of artificial insemination in the cattle breeding industries make freezing/chilling-thawing of sperm to be a routine (Graham and Moce, 2005). ROS are usually produced when this routine is carried out. Semen is exposed to cold shock and atmospheric oxygen increases the susceptibility to lipid peroxidation as a result of the higher production of ROS during cryopreservation (Bucak *et al.*, 2007). The sperm plasma membrane is one of the key structures affected by cryopreservation (Cheema *et al.*, 2009). Sperm cryopreservation and thawing is highly related with increased ROS release and decreased antioxidant level. Both freezing and thawing causes tremendous change in cell water volume. Spermatozoa discard most of their cytoplasm during the terminal stages of differentiation and lack the significant

cytoplasmic component containing antioxidants that counteract the damaging effect of ROS and LPO (Bansal and Bilaspuri 2007). Due to this, spermatozoa are susceptible to LPO during cryopreservation and thawing (Bansal and Bilaspuri 2007), which confers considerable mechanical stress on the cell membrane (Cheema *et al.*, 2009). It has been noted in humans that ROS level has a positive correlation with the extent of apoptotic sperms (Said *et al.*, 2005). Despite recent morphological advances, cryopreservation exerts detrimental effects on spermatozoa that lead to a significant decrease in sperm viability and motility, and, ultimately in decreased cryopreserved sperm rates (CSR). The fertility potential of cryopreserved mammalian spermatozoa is lower than that of fresh sperm (Chatterjee *et al.*, 2001). Frozen-thawed ram sperm demonstrates serious cryopreservation damage and thus a highly reduced fertilizing capacity (Reddy *et al.*, 2007). Long-term (freezing) and short-term (liquid) storage of sperm may lead to membrane deterioration due to membrane phase transition occurring in the regions of highly specialized sperm plasma membrane. Antioxidant capacity of semen may, however, be insufficient in preventing LPO during the freezing and chilling-thawing process. The protective antioxidant systems in sperm are primarily of cytoplasmic origin and sperm discard most of their cytoplasm during terminal stages of differentiation (Bucak *et al.*, 2008). Thus mammalian sperm lack a significant cytoplasmic component, which contains sufficient antioxidants to counteract the damaging effects of ROS and LPO. For this reason, sperm are susceptible to LPO during cryopreservation and thawing process (Bucak *et al.*, 2008). Damage due to oxidative stress may be by passed by the inclusion of antioxidants prior to freezing processes (Bucak *et al.*, 2007).

Cryopreservation induces extensive biophysical and biochemical changes in the membrane of spermatozoa that ultimately decrease the fertility potential of the cells

(Chatterjee *et al.*, 2001). Procedure of cryopreservation increases premature capacitation of spermatozoa (Ford, 2001). These alterations may not affect motility but reduces life span, ability to interact with the female reproductive tract and sperm fertility. Freezing and thawing processes also lead to the generation of reactive oxygen species (ROS) (Bucak *et al.*, 2008). Excessive production of ROS during cryopreservation has been associated with the reduced post-thaw motility, viability, membrane integrity, antioxidant status, and fertility and sperm functions. The post-thaw motility of the cryopreserved buffalo semen is poor and the success rate of IVF with buffalo sperm is only 10%–20% as compared to cattle which is 30%–35% (Reddy *et al.*, 2007).

2.26 VITAMIN C (Ascorbic acid)

Ascorbic acid (Vitamin C) is a small carbohydrate molecule which was first identified in the 1920s by a scientist called Albert von Szent Györgyi, who during his studies found that this molecule had the ability to prevent and cure scurvy (Aysun 2009). Scurvy is a pathological life-threatening condition of the bones suffered by people who do not consume fruits or vegetables for long periods of time. Ten years earlier, Kazimierz Funk prepared a list of nutritional factors which are called vitamins, deficiencies of vitamins cause severe diseases in humans. The letter "C" was used by Funk to represent a factor which was still unidentified, but known to prevent scurvy when consumed. Szent Györgyi and Haworth later chemically identified "C" as ascorbic acid, and they named it so because *ascorbic* means "anti-scurvy. Hundred years later, what we now know as vitamin C became one of the most popular drugs in the history of man (Aysun, 2009).

The sperm cells are usually exposed to oxygen and visible light radiation during the process of cryopreservation leading to the formation of ROS (Aysun, 2009). ROS production is a normal physiological event in various organs of organisms. However,

when ROS is produced in excess, it causes damage to sperm membrane, thereby reducing motility and genomic integrity of sperm cells (Baumber *et al.*, 2000). Vitamin C (Ascorbic Acid) in extenders during cryopreservation can induce optimal sperm performance by reducing cell damage due to its ability to continuously scavenge for radicals (Hu *et al.*, 2010). Ascorbic acid supplementation on standard semen quality parameters and antioxidant activities after thawing of frozen bovine semen showed that higher levels of Ascorbic acid in extenders could reduce oxidative stress provoked by freezing-thawing and improve semen quality (Aysun 2009).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study site

The study was carried out at the Artificial Insemination Unit of the National Animal Production Research Institute (NAPRI), Ahmadu Bello University, Shika-Zaria, Nigeria. Shika is located in the Northern Guinea Savannah between latitudes 11° - 12° N and between longitudes 7° E and 8° E at the elevation of 650 above sea level with an annual maximum and minimum temperature of 31 and 32° C, respectively. Shika has an average annual rainfall of 1100mm usually lasting from May to October with a mean relative humidity of 72% while the dry season lasts from November to April with mean daily temperatures ranging from 15- 36° C and mean relative humidity of between 20-37%, as described by Barje, (2006).

3.2 Experiment 1: Effect of Four different Avian Egg yolk Tri- Sodium Citrate Extenders on Bovine Semen Characteristics.

3.2.1 Experimental materials

The materials used for the experiment included

1. Microscope: for magnifying the semen,
2. Slides and cover slips and micro pipette: used for viewing samples under the microscope and dropping the semen on the slide.
3. Test tubes, conical flask and Beaker (100ml): used for preparation of diluents and measurements.
4. Haemocytometer: used in counting of cells in the semen.
5. Methylated spirit: used in sterilization of egg shell.

6. pH meter: used to check acidity and alkalinity of samples.
7. Boujour bottles: for storage of samples.
8. Detergent and cotton wool: used for cleaning.
9. Penicillin and streptomycin: used as antibiotics in diluents (extenders).
10. An artificial vagina: used for semen collection.
11. 25cm sterile whatman filter paper; egg yolk is rolled on it to remove traces of albumen to prevent harmful effect of biotin contained in it on the sperm cells.
12. Chemo craft pH paper

3.2.2 Animals and their Management

Three (3) Friesian × Bunaji bulls between 2-3 years of age were used for the experiment. The bulls were kept under intensive management system. The bulls were fed a concentrate diet at 1.5% and hay at 2.5% body weight per head per day. Water and mineral salt were provided *ad-libitum*. Animals were sprayed weekly with acaricides and any health problem was attended to regularly.

3.2.3 Experimental Treatments

Four (4) types of extenders were prepared using egg yolk from four avian species namely chicken, guinea fowl, turkey and quail.

Table 3.1: Ingredient Composition of Bulls' Diet.

Ingredients	% (Kg)
Maize	45
Wheat offal	22
Cotton seed cake	30
Bone meal	2
Salt	1
Total	100

Table 3.2: Composition of Egg Yolk Tris-Sodium Citrate Extender.

Components	Egg Yolk Extenders			
	CEYE	TEYE	QEYE	GEYE
SCB(ml)	40	40	40	40
Egg yolk(ml)	10	10	10	10
Streptomycin(mg/ml)	0.5	0.5	0.5	0.5
Penicillin(units/ml)	250	250	250	250

SCB=Sodium citrate buffer, CEYE=Chicken Egg yolk Extender, TEYE= Turkey Egg yolk Extender, QEYE= Quail Egg yolk Extender, GEYE= Guinea fowl Egg yolk Extender.

Each of the four extenders served as a treatment. The extenders were prepared according to procedure described by Rekwot *et al.*, (1997) as shown below:

Preparation of Egg yolk extenders.

1. A buffer solution was prepared by dissolving 1.45g of sodium citrate in 50ml of distilled water in a conical flask.
2. Freshly laid eggs were sterilized by cleaning the shell with methylated spirit to avoid introduction of microorganisms. The cleaned eggs were broken in half and the albumen discarded. Any trace of the albumen was removed by rolling the egg yolk carefully on a sterile 25cm filter paper.
3. 40ml of the buffer solution was put in glass tube and filled up to 50ml with 10ml of the egg yolk. 0.5ml of Streptomycin Sulphate and 0.25ml of Penicillin was added to the extender to reduce contamination by microorganisms.

3.2.4 Semen Collection and Evaluation

Semen was collected by means of an artificial vagina weekly from three bulls on each collection day. Samples from each bull was analysed for initial spermatozoa characteristics before being pooled together. Semen characteristics evaluations were as described below:

3.2.4.1 Semen Colour

The colour of each of the semen sample collected was noted/observed visually immediately after collection and recorded as watery, milky or creamy coded by 1, 2 or 3 respectively as earlier reported by Rekwot *et al.*, (1987). Stained and or watery semen was not evaluated, and had to be discarded.

3.2.4.2 Semen Volume

The semen samples were collected into transparent and calibrated rubber test tubes. The volume of each sample was read through the tubes and the value recorded in millilitres.

3.2.4.3 Sperm Motility

The gross motility was estimated immediately semen was collected from the bulls by observing a drop of semen on glass slide with cover slide under a microscope at $400 \times$ magnifications, mounted on a warm stage maintained at 37°C . A wet semen mount was made using a drop of semen placed on a microscope slide. Semen samples with 80% initial motility were used for the experiment. Gross motility was estimated as percentage score according to the procedure outlined by Zemjanis, (1971).

3.2.4.4 Semen pH

The hydrogen ion concentration (pH) of individual sample collected was determined using Chemo craft pH paper and pH meter. This is a pH paper calibrated from 1 to 14 with different colours. One inch of the paper was inserted into the semen while in the tube for five seconds, then it was removed and air dried properly. The colour change observed after drying were placed to the colour chart on the pH paper. The corresponding pH number for the colour on the chart was recorded as the pH of the sample.

Table 3.3: Descriptive and numerical scales for evaluation of gross motility of bull semen

Numerical scale	Descriptive scale	Percentage Sperm Motility	Appearance of pattern
0	Very poor	0-20%	Spermatozoa are immotile. No wave pattern
1	Poor	20-40%	Stationary bunting or weak rotary movements are exhibited by spermatozoa. No wave pattern
2	Fair	40-50%	Oscillatory or rotary movements and fewer than 50% of the spermatozoa are in motion. Barely distinguishable wave or eddies
3	Good	50-80%	Progressive rapid movement of spermatozoa with slowly moving waves and eddies. Usually 50-80% of the spermatozoa must be progressively motile to produce wave and eddies.
4	Very good	80-90%	Vigorous, progressive movement with rapid and abruptly forming waves and eddies; indicating about 90% motile spermatozoa. Dark, distinct waves.
5	Excellent	90-100%	Very vigorous forward motion. Extremely rapid waves and eddies indicating about 100% actively motile spermatozoa

Source: Zemjanis, 1971.

3.2.4.5 Sperm Concentration

The concentration of the spermatozoa was determined using the Red Blood Cell counting chamber of a haemocytometer that was crossed with microscopic grids containing 25 large squares. With each large square containing sixteen smaller squares. This was placed on a microscope and viewed with $\times 400$ objective. The total number of smaller squares on the haemocytometer is 400. Sperm cells were counted diagonally from top left to the bottom right and from top right to the bottom left in five large squares. A total of 80 smaller squares sperm counted were multiplied by a dilution factor and a multiplication factor, and value obtained was recorded as the sperm concentration (Rekwot *et al.*, 1987).

3.2.4.6 Live and Dead Spermatozoa

The live and dead sperm were estimated by preparing a smear of individual semen or semen extender sample using eosin-nigrosin stain immediately after collection or post thawing using the procedure of Hancock (1957). A drop of the diluted semen was placed on a clean glass slide using a micro-pipette. A drop of the eosin-nigrosin solution was placed alongside the semen on the slide. A gentle circular turning of the slide was done to allow a uniform mixture of the two samples. A quarter of the part of another clean slide was placed on top of the first sample and the two slides were gradually and carefully drawn apart to prepare a thin smear on the first slide. This was allowed to dry and thereafter labelled.

The principle is that the dead sperm cells pick the stain and appear pink or red while the live sperm cells repel the stain and remain unstained. This is due to the fact that the membrane of the dead sperm cells are ruptured hence picking the pink colour while that of the live sperm cells are still intact repelling the colour. Live and abnormal spermatozoa percentages were counted using a hand counter, and recorded as ratios.

3.2.4.7 Sperm Morphology

The same slides prepared for live and dead ratio were used for the morphological studies. The total number of normal and abnormal cells was counted using hand counter and recorded. Abnormalities recorded were coiled tail, detached head, mid-piece droplet and their number recorded. Other possible abnormalities are cytoplasmic droplets, tapered head, micro heads, macro heads, loose tails, amorphous heads, double heads, coiled double tail, distal cytoplasmic droplets, folded tail, double tail, cork-screw midpiece with droplet.

3.2.5 Semen Dilution and Storage

A dilution rate of 1:4 v/v (semen: diluent) was used. The dilution was done in 5ml boujour bottles. Sixteen (16) boujour bottles each containing the diluted semen using the different egg yolk extenders were stored in a refrigerator at 5⁰C over a period of 3 days and monitored or evaluated at 0, 24, 48 and 72 hours.

3.2.6 Post-Storage Semen Evaluation

At the end of each storage period, four samples, one from each extender group was taken out and warmed in a water bath at 37⁰C for 10 minutes. Samples of the thawed extender were taken out using a micropipette, placed in a glass slide and covered with a cover slip and analysed under a microscope at 100× magnification. The post storage semen characteristics determined were spermatozoa abnormalities (normal sperm, mid piece droplet, detached head, free tail, and coiled tail), motility, viability, and pH. Sperm concentration was determined by means of a spectrophotometer/Beckman model C- 4001 calibrated against haemocytometer counts at 600mM wavelength. The experiment was replicated at weekly interval over a period of 5 weeks.

3.2.7 Data analysis

Data was analysed using the Analysis of Variance (ANOVA) of Statistical Analysis System (SAS, 2002). Significant means were separated using Duncan Multiple Range Test (Duncan, 1955).

3.2.8 Experimental design

The experimental design used was a 4×4 Factorial arrangement in a Completely Randomized Design, with 4 egg yolk extenders (chicken, guinea fowl, quail and turkey) at 4 storage periods (0, 24, 48 and 72 hours) with experiments carried out for 5 weeks (replicates), respectively. The model used for the analysis is as follows:

$$Y_{ijk} = \mu + A_i + B_j + (A \times B)_{ij} + e_{ijk}$$

Where:

Y_{ijk} = Observations of the semen characteristics.

μ = Overall mean

A_i = Effect of i^{th} egg yolk extender ($i = 1, 2, 3, 4$)

B_j = Effect of j^{th} Storage Period ($j = 0, 24, 48$ and 72 hours)

$(A \times B)_{ij}$ = Interaction between Egg yolk extenders and storage periods.

e_{ijk} = Random error

3.3 Experiment 2: Effect of Vitamin C Inclusion levels in Chicken and Quail Egg yolk Tri- Sodium Citrate Extenders on Bovine Semen Characteristics.

3.3.1 Experimental materials and Animal management

As in experiment 1 except for addition of Vitamin C

3.3.2 Experimental Treatments

Six (6) types of extenders were prepared using chicken and quail egg yolk with each extender divided into three aliquots having 0, 3 and 6mg/ml Vitamin C levels.

Table 3.4: Composition of Egg Yolk Sodium Citrate Extender with varying levels of Vitamin C.

Components	Egg Yolk Extenders					
	T1	T2	T3	T4	T5	T6
SCB(ml)	40	40	40	40	40	40
Egg yolk(ml)	10	10	10	10	10	10
Streptomycin(mg/ml)	0.5	0.5	0.5	0.5	0.5	0.5
Penicillin(units/ml)	250	250	250	250	250	250
Vitamin C (mg/ml)	0	3	6	0	3	6

SCB=Sodium Citrate Buffer.

Each of the six extenders served as a treatment. The extenders were prepared according to procedure described by Rekwot *et al.*, (1987) as follows:

Preparation of Egg yolk extenders.

1. A buffer solution was prepared by dissolving 1.45g of sodium citrate in 50ml of distilled water in a conical flask.
2. Freshly laid eggs were sterilized by cleaning the shell with methylated spirit to avoid introduction of microorganisms. The cleaned eggs were broken in half and the albumen discarded. Any trace of the albumen was removed by rolling the egg yolk carefully on a sterile 25cm filter paper.
3. 40ml of the buffer solution was put in glass tube and filled up to 50ml with 10ml of the egg yolk. 0.5ml of Streptomycin Sulphate and 0.25ml of Penicillin was added to the extender to reduce contamination by microorganisms.
4. Granulated Vitamin C was added to the extender after preparation of the extenders and mixed thoroughly.

3.3.3 Semen Collection and Evaluation

Semen collection and evaluation were conducted as described in Experiment 1

3.3.4 Semen Dilution and Storage

A dilution rate of 1:4 v/v (semen: diluent) was used. The dilution was done in 5ml boujour bottles. Twenty four (24) boujour bottles each containing the diluted semen using the different egg yolk extenders were stored in a refrigerator at 5⁰C over a period of 0, 24, 48 and 72 hours.

3.3.5 Post – Storage Semen Evaluation

At the end of each storage period, six samples, one from each extender group was taken out and warmed in a water bath at 37⁰C for 10 minutes. Samples of the thawed extender were taken out using a micropipette, placed in a glass slide and covered with a cover slip and analysed under a microscope at 100× magnification. The semen characteristics determined were spermatozoa abnormalities (normal sperm, mid piece droplet, detached head, free tail, and coiled tail), motility, viability, volume and pH. Sperm concentration was determined by means of a spectrophotometer/Beckman model C- 4001 calibrated against haemocytometer counts at 600nm wavelength. The experiment was replicated at weekly interval over a period of 5 weeks.

3.3.6 Data analysis

Data analysis were as described in Experiment 1

3.3.7 Experimental design

2×3×4 factorial arrangement in a Completely Randomized Design. Two (2) egg yolk extenders (chicken and quail) with 3 inclusion levels of Vitamin C (0, 3 and 6mg/ml) at 4 storage periods (0, 24, 48 and 72 hours) with experiment carried out for 5 weeks (replicates), respectively. The model is as follows:

$$Y_{ijkl} = \mu + B_i + C_j + D_k + (B \times C)_{ij} + (B \times D)_{ik} + (C \times D)_{jk} + e_{ijkl}$$

Where:

Y_{ijk} = Observations of the semen characteristics.

μ = Overall mean

B_i = Effect of i^{th} inclusion level of Vitamin C ($i = 0, 3, 6\text{mg/ml}$)

C_j = Effect of j^{th} Storage Period ($j = 0, 24, 48, 72$ hours)

D_k = Effect of k^{th} Egg yolk Extenders ($k=1, 2$)

$(B \times C)_{ij}$ = Interaction between Vitamin C inclusion levels and Storage Periods.

$(B \times D)_{ik}$ = Interaction between Vitamin C inclusion levels and Egg yolk Extenders.

$(C \times D)_{jk}$ = Interaction between Storage Periods and Extenders.

$(B \times C \times D)_{ijk}$ = Interaction between Vitamin C levels, Egg yolk Extenders and Storage Periods.

e_{ijkl} = Random error

CHAPTER FOUR

4.0 RESULTS

4.1 Experiment 1: Effect of Four different Avian Species Egg yolk Tri- Sodium Citrate Extender on Bovine Semen Characteristics.

Table 4.1.1 shows the average values of the characteristics of pooled fresh bull semen used in this study. Gross motility ranged between 90 and 95% with an average of $92 \pm 0.5\%$. sperm concentration ranged from 314 to 649×10^6 cells per ml with an average of 462.3 ± 30.6 average spermatozoa viability was $81 \pm 2.7\%$ ranging from 60 to 95%, while pH averaged 6.2 ranging between 6 to 7. Mean percentage morphologically normal was $78 \pm 1.8\%$ but ranged from 64 to 88%

Table 4.1.2 shows the effect of egg yolk extenders on motility of chilled bull semen at different storage periods. There was a general decline in sperm motility across treatments (egg yolk extenders), with increase in storage period. However, the decline varied with the type of extender ranging from 88 to 66% in chicken egg yolk extender and 82 to 46% in turkey egg yolk extender. Sperm motility was significantly higher ($P < 0.05$) in the chicken egg yolk extenders than other extenders after dilution at first day of storage. Quail egg yolk extender retained significantly ($P < 0.05$) high sperm motility than guinea fowl egg yolk extender and turkey egg yolk extender in preserving sperm across the storage period. There was no significant difference ($P > 0.05$) in motility of sperm stored in guinea fowl and turkey egg yolk extender.

Table 4.1.1: Summary of initial bull semen characteristics.

Characteristics	Mean±SE	Coefficient of Variation (%)	Minimum	Maximum
Pooled semen volume(ml)	7.8±1.6	38.9	2.4	14.5
Gross motility (%)	92±0.5	2.7	90	95
Semen pH	6.2	6.58	6	7
Sperm Concentration($\times 10^6$)	462.3±30.6	33.3	314	649
Viability (%)	81±2.7	16.6	60	95
Sperm Morphology (%)				
MPD	0±0.1	223.6	0	1
DH	9±0.7	36.9	4	13
FT	3±0.6	120.4	0	7
CT	4±0.4	53.0	2	7
BT	6±1.1	92.1	0	15
NS	78±1.8	11.6	64	88

Standard Error = SE, Normal Sperm =NS, Free Tail=FT, Mid Piece Droplet =MPD, Detached Head=DH, Coiled Tail=CT and Bent Tail=BT.

Table 4.1.2:Effect of Egg yolk extender Sperm Motility of Chilled Bull semenat different Storage Periods.

Extenders	Storage Period (hours)			
	0	24	48	72
CEYE	88.0 ^a	78.0 ^a	72.0 ^a	66.0 ^a
GEYE	82.0 ^c	72.0 ^b	59.0 ^c	55.0 ^c
QEYE	85.0 ^b	71.0 ^b	66.0 ^b	60.0 ^b
TEYE	82.0 ^c	70.0 ^c	50.0 ^d	46.0 ^d
SEM	2.97	2.97	2.97	2.97
LOS	*	*	*	*

^{abcd}Means within the same column with different superscript are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance., EYE=Chicken Egg Yolk Extender, TEYE= Turkey Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, GEYE= Guinea fowl Egg Yolk Extender,

Table 4.1.3 shows the effect of egg yolk extenders on viability of sperm cells in chilled bull semen at different storage periods. There was significant ($P < 0.05$) decline in percentage viable sperms across treatments with increase in storage period. However, at 0 hour of storage percentage of viable sperm cells were higher ($P < 0.05$) in quail egg yolk extender, than in the other extenders. Percentage viable sperm cells were significantly ($P < 0.05$) higher in chicken egg yolk extender, than in guinea fowl egg and turkey egg yolk extender which were not significantly different ($P > 0.05$). There was a sharp decline in percentage viable sperm cells in the quail egg yolk extender after 24 hours storage (from 73 to 87%) compared to the other extenders. At 48 hours, the least percentage viable sperm cells was in semen stored in TEYE, while the values were similar in semen stored in chicken, guinea fowl and quail egg yolk extenders. After 72 hours of storage, chicken egg yolk extender was superior (having 71% viable sperms) to other extenders in preserving sperm followed by quail egg yolk extender.

Table 4.1.4 shows the effect of egg yolk extenders on pH of chilled bull semen at different storage periods. There was no significant difference ($P > 0.05$) in the pH semen stored in the different egg yolk semen extenders with increase in storage period. After dilution, semen samples stored in guinea fowl egg yolk extender had significantly ($P < 0.05$) pH than samples stored in other extenders. There was decline in pH of all semen samples in the various extenders after 24 hours storage, although the difference was not significantly higher ($P > 0.05$). There was a slight increase though not significant in semen pH in all extenders between 24-48 hours of storage, the pH remained constant ranging from 6.2-6.4 between 48-72 hours of storage across treatments.

Table 4.1.3:Effect of different Egg yolk extenders on Viability of sperm in of Chilled Bull semenat different Storage Periods.

Extenders	Storage Period (hours)			
	0	24	48	72
CEYE	79.0 ^{bc}	77.0 ^{ab}	72.0 ^a	71.0 ^a
GEYE	76.0 ^c	73.0 ^{abc}	72.0 ^a	54.0 ^c
QEYE	87.0 ^a	73.0 ^{abc}	72.0 ^a	63.0 ^b
TEYE	76.0 ^c	71.0 ^c	68.0 ^b	54.0 ^c
SEM	2.13	2.13	2.13	2.13
LOS	*	*	*	*

^{abcd}Means within the same column with different superscript are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance, CEYE=Chicken Egg Yolk Extender, TEYE= Turkey Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, GEYE= Guinea fowl Egg Yolk Extender.

Table 4.1.4:Effect of different Egg yolk extender on pH of Chilled Bull semen at different Storage Periods.

Extenders	Storage Period (hours)			
	0	24	48	72
CEYE	6.4 ^b	6.2	6.4	6.4
GEYE	6.8 ^a	6.0	6.2	6.2
QEYE	6.2 ^b	6.0	6.2	6.2
TEYE	6.4 ^b	6.0	6.4	6.4
SEM	0.1	0.1	0.1	0.1
LOS	*	NS	NS	NS

^{ab}Means within the same column with different superscript are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance CEYE=Chicken Egg yolk Extender, TEYE= Turkey Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, GEYE= Guinea fowl Egg Yolk Extender.

Table 4.1.5 shows the effect of egg yolk extenders on percentage of normal sperm cells in chilled bull semen at different storage periods. There was a general but not significant ($P>0.05$) decline in percentage of normal sperm cells across treatments with increase in storage period, ranging from 86 to 81% in QEYE and TEYE, 85 to 78% in GEYE and 84 to 82% in CEYE. However, across treatments, there were slightly but not significant difference in percentage normal cells with increase in storage time except in GEYE which had a significantly ($P<0.05$) low percentage sperm cells after 72 hours storage than in semen stored in other extenders.

Table 4.1.6 shows the occurrence of abnormalities in chilled bull spermatozoa stored in various avian egg yolk extenders. There was a significant ($P<0.05$) increase in percentage sperm abnormalities with increase in length of storage. Across treatments (egg yolk types), DH was slightly higher in semen samples stored in QEYE and TEYE, higher in CEYE and GEYE and higher in TEYE after 24, 48 and 72 hours of storage respectively. However, these differences were not significant. FT was significantly higher ($P<0.05$) in semen samples stored in GEYE after 72 hours storage than in the other diluents. Similarly, BT was significantly ($P<0.05$) higher in semen samples stored in TEYE than in those stored in other diluents (extenders) after 72 hours of storage. Slight but not significant differences were observed in BT across treatments.

Table 4.1.5:Effect of different Egg yolk extenderon Percentage Normal Sperm in Chilled Bull semenat different Storage Periods.

Extenders	Storage Period (hours)			
	0	24	48	72
CEYE	84.0	84.0	84.3	82.0 ^a
GEYE	85.0	83.0	82.0	78.0 ^c
QEYE	86.0	83.0	81.0	81.0 ^{abc}
TEYE	86.0	84.0	82.0	81.0 ^{abc}
;SEM	1.9	1.9	1.9	1.9
LOS	NS	NS	NS	*

^{abcd}Means within the same column with different superscript are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance. CEYE=Chicken Egg Yolk Extender, TEYE= Turkey Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, GEYE= Guinea fowl Egg Yolk Extender.

Table 4.1.6: Effect of different Egg yolk extender on Percentage Sperm abnormalities of Chilled Bull semen at different Storage Times.

Abnormalities	Extenders	Storage Time (hours)				SEM
		0	24	48	72	
MPD	CEYE	0.0	0.0	0.0	0.0	0.04
	GEYE	0.0	0.0	0.0	0.0	
	QEYE	0.0	0.0	0.0	0.0	
	TEYE	0.0	0.0	0.0	0.0	
DH	CEYE	6.0 ^a	6.0 ^b	9.0 ^a	8.0 ^b	1.18
	GEYE	5.0 ^b	6.0 ^b	9.0 ^a	9.0 ^a	
	QEYE	6.0 ^a	7.0 ^a	7.0 ^b	8.0 ^b	
	TEYE	5.0 ^b	7.0 ^a	7.0 ^b	10.0 ^a	
FT	CEYE	2.0 ^c	3.0 ^b	4.0 ^b	6.0 ^b	0.66
	GEYE	3.0 ^b	3.0 ^b	5.0 ^a	8.0 ^a	
	QEYE	3.0 ^b	3.0 ^b	4.0 ^b	5.0 ^c	
	TEYE	4.0 ^a	4.0 ^a	4.0 ^b	5.0 ^c	
CT	CEYE	1.0 ^a	1.0 ^b	1.0 ^b	2.0 ^b	0.42
	GEYE	0.0 ^b	1.0 ^b	1.0 ^b	3.0 ^a	
	QEYE	1.0 ^a	2.0 ^a	2.0 ^a	2.0 ^b	
	TEYE	1.0 ^a	1.0 ^b	2.0 ^a	2.0 ^b	
BT	CEYE	4.0 ^a	4.0 ^a	4.0 ^{ab}	5.0 ^b	0.77
	GEYE	3.0 ^{ab}	4.0 ^a	5.0 ^a	6.0 ^{ab}	
	QEYE	3.0 ^{ab}	4.0 ^a	5.0 ^a	5.0 ^b	
	TEYE	3.0 ^{ab}	3.0 ^{ab}	4.0 ^{ab}	8.0 ^a	

^{abcd}Means within the same column with different superscript are significantly different ($P < 0.05$). CEYE=Chicken Egg Yolk Extender, TEYE= Turkey Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, GEYE= Guinea fowl Egg Yolk Extender, SEM= Standard Error of Mean, LOS= Level of Significance. Normal Sperm =NS, Free Tail=FT, Mid piece Droplet =MPD, Detached Head=DH, Coiled Tail=CT and Bent Tail

4.2 Experiment 2: Effect of Vitamin C Inclusion levels in Chicken and Quail Egg yolk Tri- Sodium Citrate Extenders on Bovine Semen Characteristics.

Table 4.2.1 shows the average values of the characteristics of pooled fresh semen used in the second study. Gross motility ranged between 80 and 85% (average $89 \pm 7.3\%$). Sperm concentration ranged between 294 and 398×10^6 cells per ml (average 357 ± 9.3). Average spermatozoa viability was $88 \pm 1.14\%$ ranged from 80-95%, while pH ranged from 6 to 7 with average 6.6 ± 0.1 . Mean morphologically normal cells were $95 \pm 0.4\%$ ranging from 80 to 92%

The effects of level of Vitamin C inclusion in the Tris-egg yolk extenders on the motility of chilled bull spermatozoa are shown in table 4.2.2 and Figure 1. There was significant ($P < 0.05$) with increase in the level of Vitamin C in the extender (Figure 1 and 2). Although there were no significant ($P > 0.05$) differences in sperm motility between semen extenders across and after 24 hours storage, motility was slightly higher in QEYE than in CEYE. The same trend was maintained over storage period, with motility becoming significantly ($P < 0.05$) higher in the QEYE than CEYE across extenders. However, motility values for extenders with Vitamin C were significantly ($P < 0.05$) higher than in the extender without VitaminC.

Table 4.2.3 shows viability of chilled bull spermatozoa stored in semen extenders at various levels of Vitamin C. the result shows a general , though not significant ($P > 0.05$) increase in percentage of viable spermatozoa with increase in the levels of Vitamin C across the period (Figure 3 and 4). However, sperm viability declined significantly ($P < 0.05$) with increase in storage period. From 82 to 64% and 82 to 59% in samples stored CEYE and QEYE without Vitamin C respectively. After 72 hours of storage, similarly sperm viability decline from 80 to 64% and 80 to 67% in CEYE and QEYE

with 3mg/ml Vitamin C respectively and from 80 to 70% in CEYE and QEYE extenders with 6mg/ml respectively after 72 hours of storage. Within storage period, there was no significant ($P>0.05$) difference in viability between CEYE and QEYE diluents regardless of the level of Vitamin C.

Table 4.2.4 shows the effect of vitamin C on pH of chilled bull semen in chicken and quail egg yolk extenders at different storage periods. There was generally no significant difference in pH of chicken and quail egg yolk extenders with varying levels of vitamin C during the storage period. At 0 and 24 hour, pH was maintained between 6.3-6.4. After 48 hours, the pH ranged between 6.3-6.5 with QEYE having 6mg/ml vitamin C having significantly ($P<0.05$) the highest pH. However, after 72 hour the pH dropped and it ranged between 6.3-6.4.

Table 4.2.1: Summary of initial bull semen characteristics.

Characteristics	Mean±SE	Coefficient of Variation (%)	Minimum	Maximum
Pooled semen volume(ml)	7.5±0.2	4.9	5	14
Gross motility (%)	89±1.3	7.3	95	80
Semen pH	6.8±0.1	6.6	6	7
Sperm Concentration(×10 ⁶)	357±9.3	13	294	398
Viability (%)	88±1.14	6.5	80	95
Sperm Morphology (%)				
MPD	0±0.1	223	0	1
DH	1±0.2	104	0	1
FT	1±0.2	81.4	0	3
CT	1±0.1	39.1	1	2
BT	2±0.2	46.5	1	3
NS	95±0.4	1.9	92	96

Standard Error = SE, Normal Sperm (NS), Free Tail (FT), Mid Piece Droplet (MPD), Detached Head (DH), Coiled Tail (CT) and Bent Tail (BT)

Table 4.2.2:Effect of Vitamin C levels on Sperm Motility of Chilled Bull semenin Chicken and Quail Egg yolk extender at different Storage Periods.

Storage period(hours)	Extenders	Vitamin C Level(mg/ml)		
		0	3	6
0	CEYE	82.0	81.0	80.0
	QEYE	82.0	82.0	80.0
	SEM	1.6	1.6	1.6
	LOS	NS	NS	NS
24	CEYE	71.0 ^b	74.0	75.0
	QEYE	76.0 ^a	77.0	77.0
	SEM	1.6	1.6	1.6
	LOS	*	NS	NS
48	CEYE	65.0 ^b	67.0 ^b	69.0 ^b
	QEYE	70.0 ^a	72.0 ^a	75.0 ^a
	SEM	1.6	1.6	1.6
	LOS	*	*	*
72	CEYE	56.0 ^b	56.0 ^b	65.0 ^b
	QEYE	60.0 ^a	70.0 ^a	70.0 ^a
	SEM	1.6	1.6	1.6
	LOS	*	*	*

^{ab}Means within the same columns in the same storage period with different superscripts are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance. CEYE=Chicken Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, V/C= Vitamin C

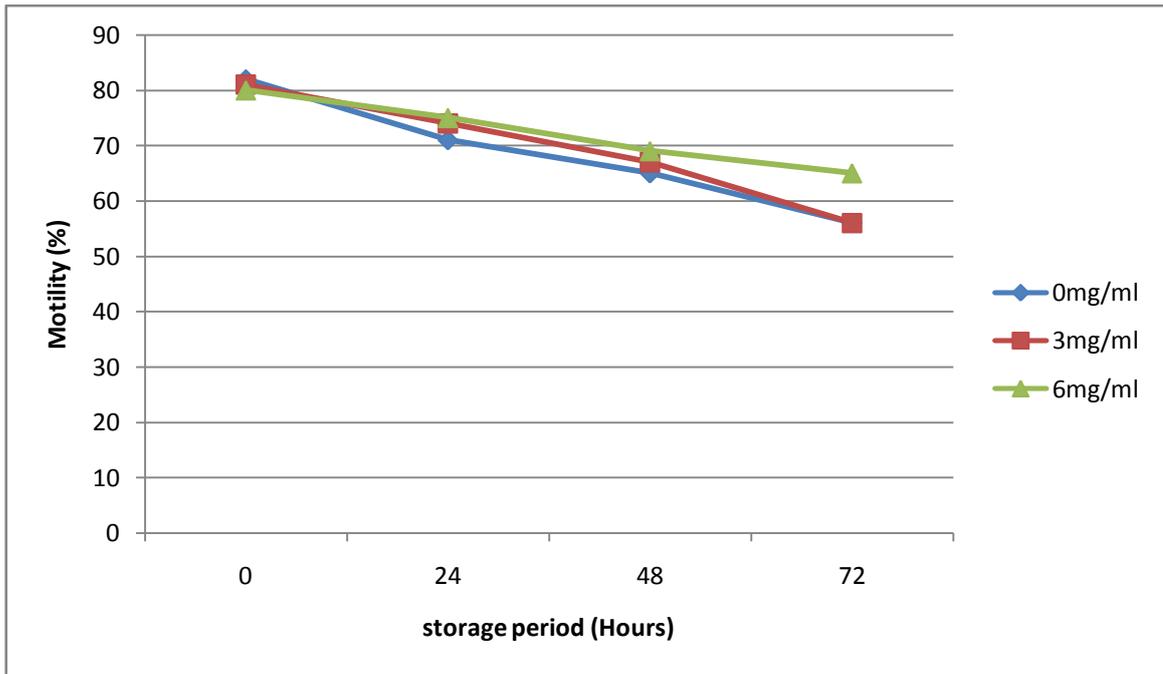


Figure 1: Effect of Vitamin C levels in chicken egg yolk extender on sperm motility in chilled bull semen

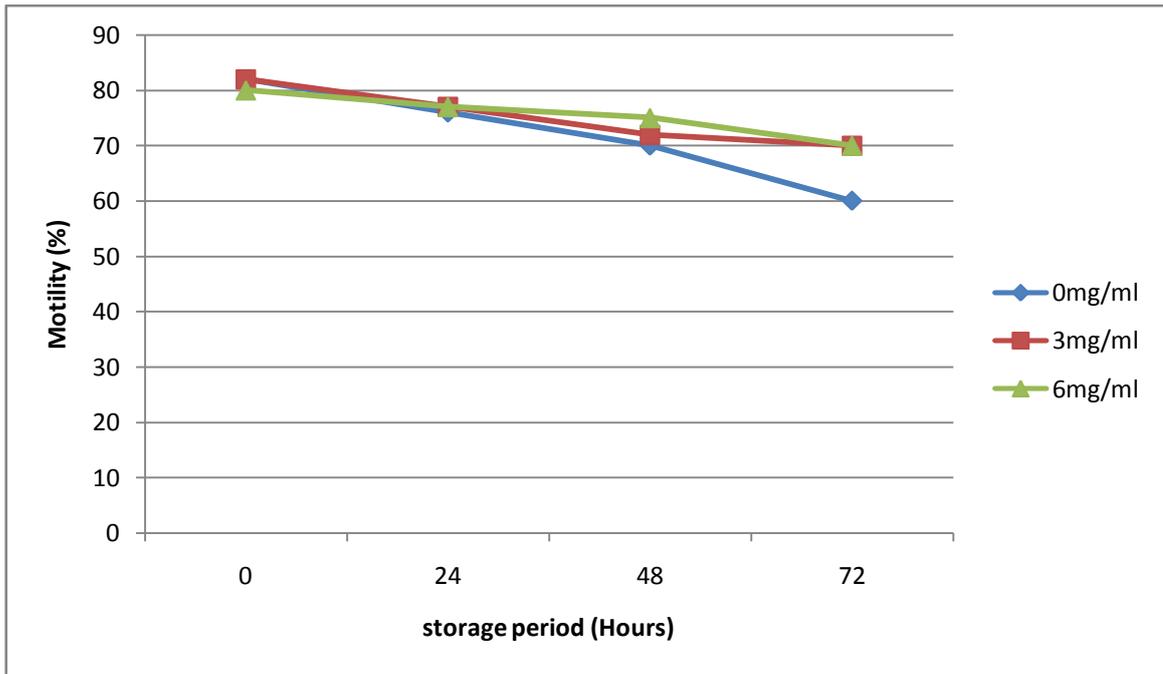


Figure 2: Effect of Vitamin C levels in quail egg yolk extender on sperm motility in chilled bull semen

Table 4.2.3:Effect of Vitamin C levels on Sperm Viability of Chilled Bull semen in Chicken and Quail Egg yolk extender at different Storage Periods.

Storage period(hours)	Extenders	Vitamin C Level(mg/ml)		
		0	3	6
0	CEYE	82.0	80.0	80.0
	QEYE	82.0	80.0	85.0
	SEM	2.3	2.3	2.3
	LOS	NS	NS	NS
24	CEYE	72.0	74.0	76.0
	QEYE	72.0	74.0	73.0
	SEM	2.3	2.3	2.3
	LOS	NS	NS	NS
48	CEYE	68.0	68.0	69.0
	QEYE	68.0	72.0	72.0
	SEM	2.3	2.3	2.3
	LOS	NS	NS	NS
72	CEYE	61.0	64.0	66.0
	QEYE	59.0	67.0	70.0
	SEM	2.3	2.3	2.3
	LOS	NS	NS	*

^{abcd}Means within the same column with different superscripts are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance. CEYE=Chicken Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, V/C= Vitamin C

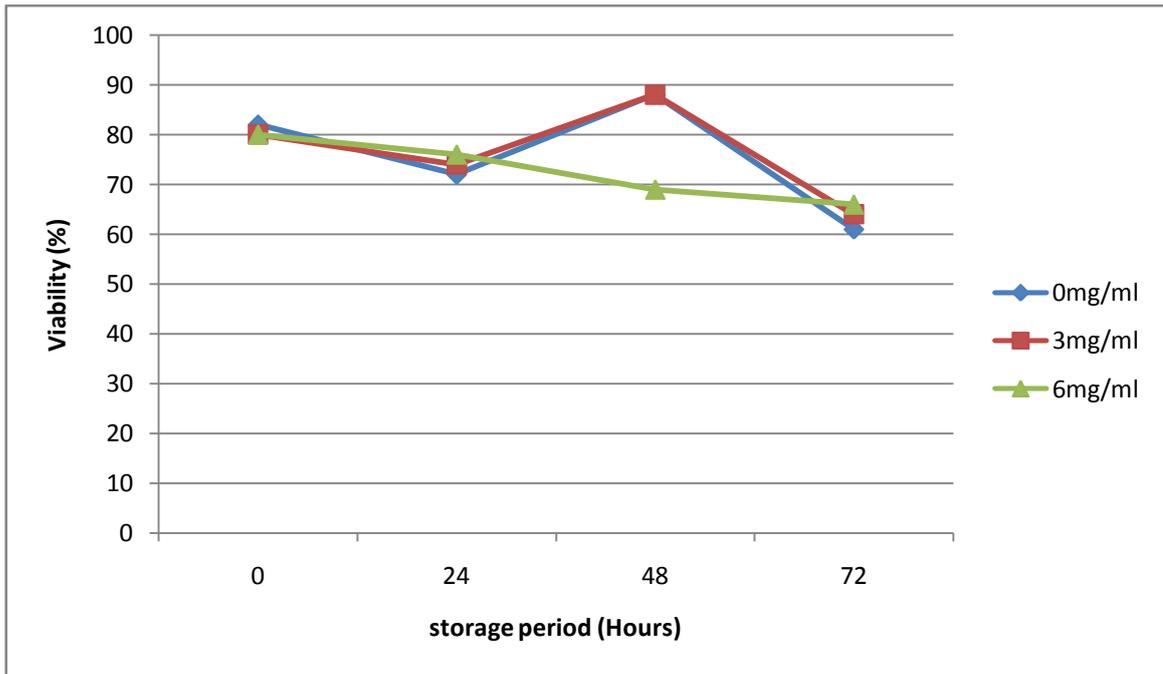


Figure 3: Effect of Vitamin C levels in chicken egg yolk extender on sperm viability in chilled bull semen

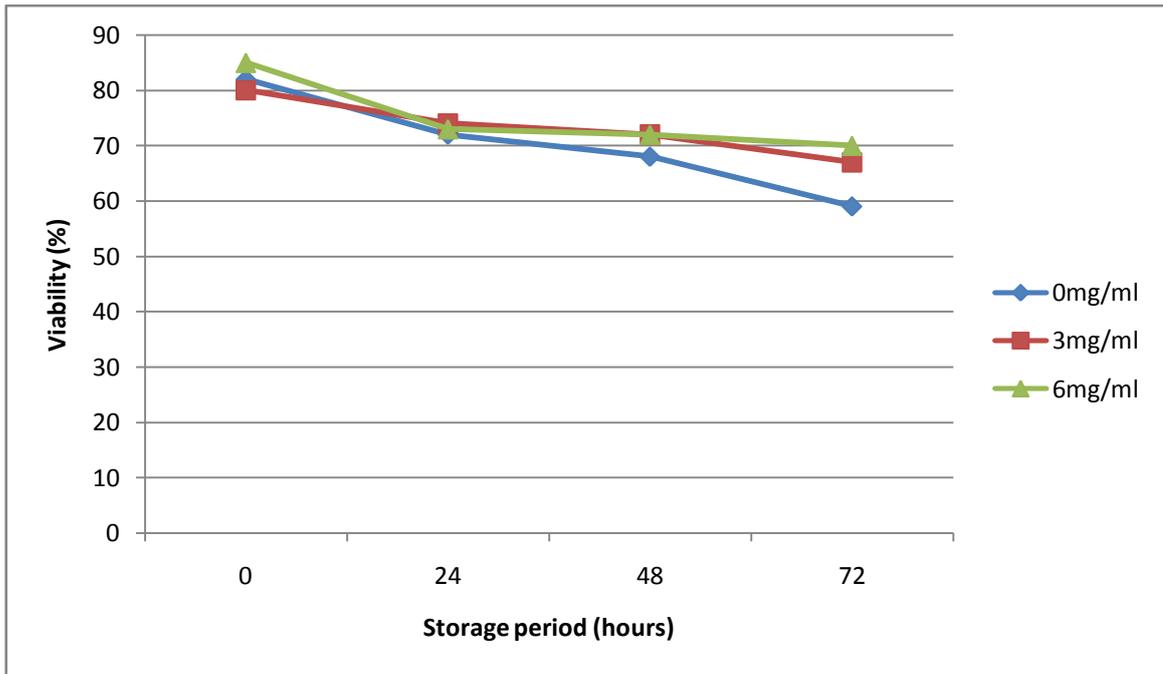


Figure 4: Effect of Vitamin C levels in quail egg yolk extender on sperm viability in chilled bull semen

Table 4.2.4:Effect of Vitamin C levels on pH of Chilled Bull semenin Chicken and Quail Egg yolk extender at different Storage Periods.

Storage period(hours)	Extenders	Vitamin C Level(mg/ml)		
		0	3	6
0	CEYE	6.4	6.4	6.4
	QEYE	6.4	6.4	6.3
	SEM	0.1	0.1	0.1
	LOS	NS	NS	NS
24	CEYE	6.4	6.4	6.3
	QEYE	6.4	6.4	6.4
	SEM	0.1	0.1	0.1
	LOS	NS	NS	NS
48	CEYE	6.4	6.4	6.3
	QEYE	6.4	6.4	6.5
	SEM	0.1	0.1	0.1
	LOS	NS	NS	NS
72	CEYE	6.4	6.4	6.4
	QEYE	6.4	6.4	6.3
	SEM	0.1	0.1	0.1
	LOS	NS	NS	NS

^{abcd}Means within the same column with different superscripts are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance. CEYE=Chicken Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, V/C= Vitamin C

Table 4.2.5 shows the effect of vitamin C on percentage normal sperm cells of chilled bull semen in chicken and quail egg yolk extenders at different storage periods. There was a general but not ($P>0.05$) significant decline in percentage of normal cells with increase in storage time. There was generally no significant ($P<0.05$) decline in percentage of normal sperm cells with increase in Vitamin C levels in both chicken and quail egg yolk extenders across storage periods (Figures 5 and 6). At 0 hours, there was a range of 88-90% normal sperm cells amongst the treatments. After 24 hours, there was no significant ($P>0.05$) decline in the percentage of normal cells in the extenders. CEYE and QEYE control treatment and 3mg/ml had no significant ($P>0.05$) difference but there was significant difference between both extenders having 6mg/ml vitamin C. however, QEYE with 3mg/ml vitamin C had significantly ($P<0.05$) highest percentage of viable cells (88%) and CEYE with 3 and 6mg/ml vitamin C had significantly ($P<0.05$) the least viable sperm cell.

At 48 hours, the range was between 83-85% and there was no significant ($P>0.05$) difference between treatments with the different levels of Vitamin C. after 72 hours, there was NO significant ($P>0.05$) decline in percentage of normal sperm cells in all the extenders ranging between 74-84% with CEYE and QEYE having 6mg/ml vitamin C with significantly ($P<0.05$) the highest percentage of normal sperm cells (84%) and QEYE control treatment having significantly ($P<0.05$) the least percentage of normal cells (74%).

Table 4.2.5:Effect of Vitamin C levels on Percentage Normal Sperm Cells of Chilled Bull semen in Chicken and Quail Egg yolk extender at different Storage Periods.

Storage period(hours)	Extenders	Vitamin C Level(mg/ml)		
		0	3	6
0	CEYE	90.0 ^a	86.0 ^b	88.0
	QEYE	88.0 ^b	90.0 ^a	90.0
	SEM	1.6	1.6	1.6
	LOS	NS	*	NS
24	CEYE	87.0	84.0	84.0
	QEYE	85.0	88.0	87.0
	SEM	1.6	1.6	1.6
	LOS	NS	NS	NS
48	CEYE	85.0	83.0	83.0
	QEYE	84.0	84.0	85.0
	SEM	1.6	1.6	1.6
	LOS	NS	NS	NS
72	CEYE	75.0	82.0	84.0
	QEYE	74.0	81.0	84.0
	SEM	1.6	1.6	1.6
	LOS	NS	NS	NS

^{abcd}Means within the same column with different superscripts are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance. CEYE=Chicken Egg Yolk Extender, QEYE= Quail Egg Yolk Extender, V/C= Vitamin C

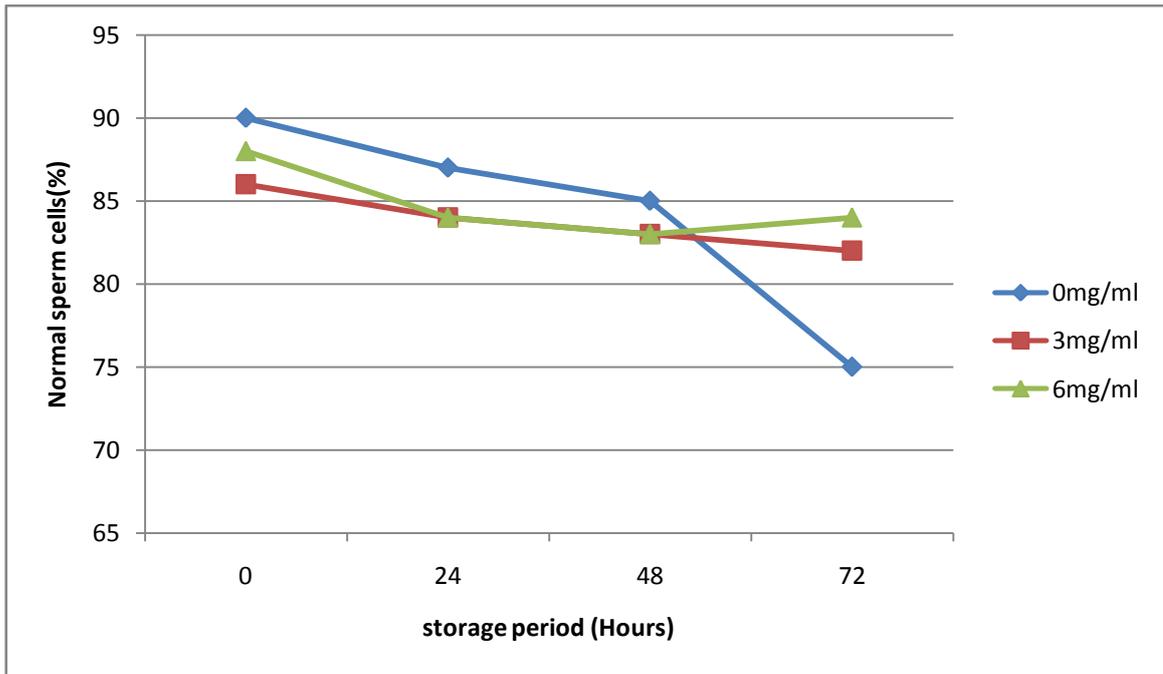


Figure 5: Effect of Vitamin C levels in chicken egg yolk extender percentage normal sperm cells in chilled bull semen

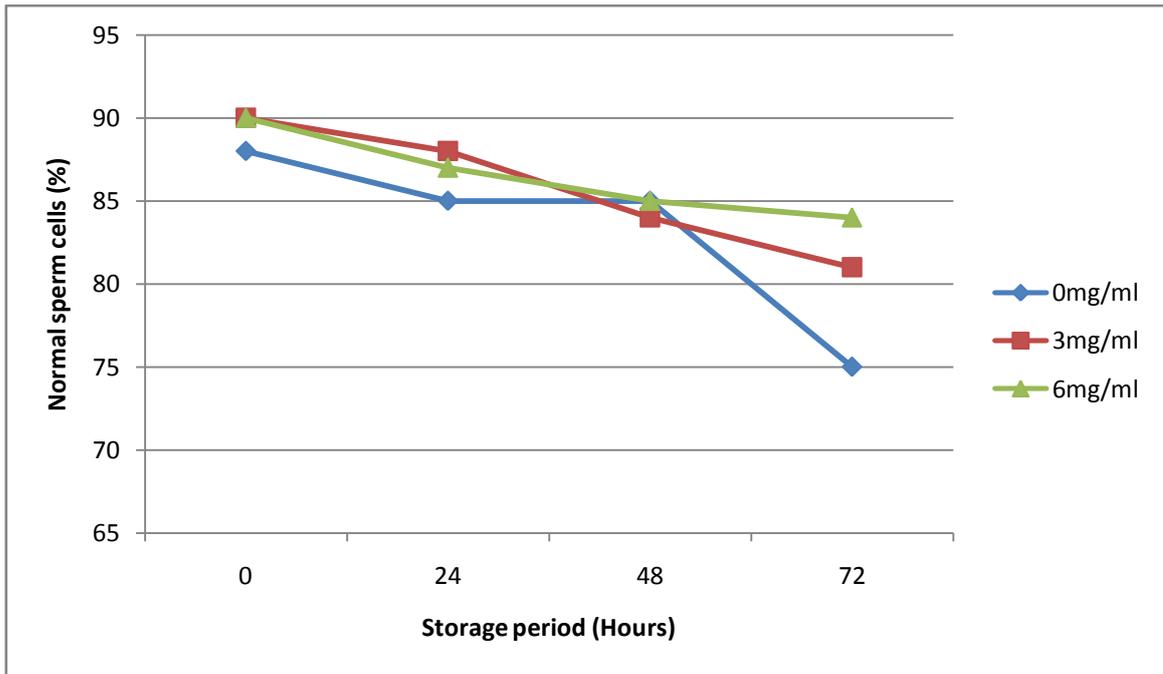


Figure 6: Effect of Vitamin C levels in quail egg yolk extender percentage normal sperm cells in chilled bull semen

The effect of Vitamin C levels on percentage sperm abnormalities of chilled bull semen in chicken and quail egg yolk extender at different storage periods is shown in table 4.2.6 and 4.2.7. This result shows that there was a significant ($P < 0.05$) increase in percentage of abnormalities with increase in storage time but there was no significant ($P > 0.05$) increase in percentage of abnormalities with increase in levels of Vitamin C. The result shows that there was no significant difference ($P > 0.05$) on MPD (Mid piece droplet), DH (Detached head), FT (Free tail), CT (Coiled tail) and BT (Bent tail).

Table 4.2.6: Effect of Vitamin C levels on Percentage Sperm Abnormalities of Chilled Bull semen in Chicken and Quail Egg yolk extender at different Storage Periods.

ST	ABN	MPD							DH							FT						
	E+V(mg/ml)	C0	C3	C6	Q0	Q3	Q6	LOS	C0	C3	C6	Q0	Q3	Q6	LOS	C0	C3	C6	Q0	Q3	Q6	LOS
0		0	0	0	0	1	0	NS	3.0 ^b	5.0 ^a	2.0 ^{ac}	5.0 ^a	3.0 ^b	3.0 ^b	*	4.0 ^b	5.0	2.0	2.0 ^a	4.0 ^b	4.0 ^b	*
24		0	0	0	0	0	0	NS	4.0 ^{ab}	6.0 ^a	5.0 ^b	5.0 ^b	5.0 ^b	5.0 ^b	*	6.0	6.0	5.0	4.0 ^b	5.0 ^{ab}	5.0 ^{ab}	NS
48		0	0	0	0	0	0	NS	5.0 ^c	7.0 ^a	5.0 ^c	6.0 ^b	5.0 ^c	4.0 ^{cd}	*	7.0	6.0	6.0	5.0	5.0	5.0	NS
72		0	0	0	0	0	0	NS	5.0 ^{cd}	9.0 ^a	6.0	8.0 ^{bc}	6.0 ^c	8.0 ^b	*	7.0	9.0 ^a	7.0 ^b	6.0	6.0	7.0	*
SEM		0.09							0.10							0.68						

^{abcd}Means within the same row with different superscripts are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance, Free Tail=FT, Mid Piece Droplet =MPD, Detached Head=DH. V= Vitamin C, C= Chicken Egg Yolk Extender, Q= Quail Egg Yolk Extender, ST= Storage Times, ABN= Sperm Abnormalities, E= Extenders

Table 4.2.7: Effect of Vitamin C levels on Percentage Sperm Abnormalities of Chilled Bull semen in Chicken and Quail Egg yolk extender at different Storage Periods.

ST	ABN	CT							BT						
	E+V(mg/ml)	C0	C3	C6	Q0	Q3	Q6	LOS	C0	C3	C6	Q0	Q3	Q6	LOS
0		0.0	0.0	0.0	0.0	0.0	0.0	NS	2.0	2.0	3.0	3.0	2.0	2.0	NS
24		0.0	1.0	0.0	0.0	0.0	0.0	NS	3.0	3.0	4.0	3.0	2.0	3.0	NS
48		1.0	1.0	1.0	0.0	0.0	0.0	NS	3.0	4.0	4.0	5.0	2.0	3.0	NS
72		2.0	1.0	1.0	2.0	0.0	1.0	NS	4.0	4.0	6.0	7.0	3.0	4.0	NS
SEM		0.68							0.64						

^{abcd}Means within the same row with different superscripts are significantly different (P<0.05). SEM= Standard Error of Mean, LOS= Level of Significance Free. Coiled Tail=CT and Bent Tail=BT, ABN= Sperm Abnormalities, E= Extenders, V= Vitamin C, C= Chicken Egg Yolk Extender, Q= Quail Egg Yolk Extender

CHAPTER FIVE

5.0 DISCUSSION

5.1 **Experiment 1: Effect of four different avian egg yolks in tris- sodium citrate extender on bovine semen characteristics**

The result of this study shows that chicken egg yolk had the highest motility while turkey egg yolk had the least after 72 hours of storage. This result agrees with Kalaksiz *et al.*, (2010) and Rauch, (2013) who reported that bull and ram sperm motility was poorest in turkey egg yolk extenders compared to other egg yolks used. This may be due to its dense egg yolk particles (Nagy *et al.*, 2003). However, it disagrees with Burris and Webb, (2009) who reported that inclusion of turkey egg yolk in semen extender produced the highest post thaw sperm motility than three other egg yolks which include chicken egg yolk, while Akhter *et al.*, (2010) reported that chicken egg yolk had least sperm motility in Sawhili bulls when compared to turkey, guinea fowl and pigeon egg yolks post thawing. Motility is a strong indicator of spermatozoa quality during semen evaluation that is why it is widely used in fertility laboratories. Studies show that reduction in semen cholesterol was observed in spermatozoa after the cryopreservation cycle, which correlated with an increased number of acrosome-reacted cells and changes in the parameters of motility in boar spermatozoa (Maldjian *et al.*, 2005). In general, studies have shown that phospholipids, cholesterol and low density lipoproteins in egg yolk may be the factors that provide protection to sperm against cold shock during cooling- thaw process of cryopreservation (Anton *et al.*, 2006; Kalaksiz *et al.*, 2010). It has been suggested that avian species and even breeds of species have different combinations of these factors (Burris and Webb, 2009). This may be the reason behind the discrepancies in motility of the spermatozoa in the different avian egg yolks.

The four avian egg yolk extenders had significantly different effect on viability of bovine spermatozoa. After 72 hours, chicken egg yolk extender had significantly higher number of viable sperm cells followed by quail egg yolk. However, guinea fowl and turkey egg yolk extenders had significantly the same sperm viability. This result is similar to the findings of Su *et al.*, (2008) and Kalaksiz *et al.*, (2010) who reported that turkey egg yolk extender had the least viable sperm cells when evaluating ram and bovine semen characteristics stored in different avian specie egg yolk extender. However, Ibrahim *et al.*, (2012) reported duck egg yolk had more viable sperm cells than chicken egg yolk when used to preserve buffalo bull semen and Gholami *et al.*, (2012) when evaluating the effect of egg yolk of four avian species on cryopreserved ram spermatozoa. Viable cells are the percentage of cells that are alive in the semen when viewed under a microscope and this is assessed by staining a drop of semen extender with eosin-nigrosin stain making a thin smear on a glass slide and allowing it to dry before viewing under the microscope at magnification 100× (Rekwot *et al.*, 1997).

Due to cold shock during cryopreservation, the sperm cell gets nourishment and protection of acrosome and plasma membrane from the cholesterol phospholipids and low density lipoprotein components of the egg yolk hence increasing their viability (Foote, 2002). Egg yolk phospholipid detoxifies the seminal plasma by grabbing the harmful proteins in it thereby increasing the viability of sperm cell (Bathgate *et al.*, 2006). These factors may be the reason for improved viability in sperm cells.

The result shows that there was no significant difference on the pH of the semen extenders. This result agrees with Foote *et al.*, (2002) and Vishwanath, (2003) who reported that pH of bull semen extenders ranges from 6-7. pH of the semen is the hydrogen ion concentration of the semen which indicates the degree of acidity and

alkalinity of the semen and it ranges from 1-14 respectively. However, when there is incomplete ejaculation, pathogenic situation of the testis or over usage of young bulls the pH could drop making it acidic (Foote, 2002).

Although there was no significant difference in the effects of the four egg yolk extenders on per cent normal sperm cells across storage time, after 72 hours, chicken egg yolk extender had significantly higher normal cells, followed by quail egg yolk and turkey egg yolk with guinea fowl egg yolk extender having the least.

There was no significant difference between the egg yolk on the MPD of the spermatozoa but there was significant difference in DH, FT, CT and BT after 72 hours of storage with more sperm abnormalities observed in turkey egg yolk and guinea fowl egg yolk than chicken egg yolk and quail egg yolk respectively. This result disagrees with that of Su *et al.*, (2008), Akhter *et al.*, (2012) and Gholami *et al.*, (2012) who reported that chicken among other avian species egg yolk during cryopreservation of bull and ram had more abnormalities and less normal cells.

Any morphological deviation from the normal structure of the spermatozoa is regarded as an abnormality (Akhter *et al.*, 2012). A report by Maldjian *et al.*, (2005) suggested that spermatozoa were damaged significantly by the cryopreservation cycle hence increasing percentage of head, neck and tail abnormalities. Low density protein of egg yolk is proposed to be the main factor for protecting spermatozoa against abnormalities, and it does this by stabilizing the membrane phospholipid losses and engulfing any harmful seminal plasma protein (Manjunath *et al.*, 2002).

5.2 Experiment 2: Effect of Vitamin C inclusion levels in chicken and quail egg yolk Tris- sodium citrate extenders on bovine semen characteristics

The result of this study shows that there was significant difference in the motility of the spermatozoa in the two egg yolk extenders each having Vitamin C included at three levels. However after 72 hours it was shown that quail egg yolk extender with 6mg/ml Vitamin C and 3mg/ml had the highest rate of motility followed by chicken egg yolk extender with 6mg/ml Vitamin C inclusion. The least was the chicken egg yolk extender control with 56% motility. The result agrees with Hu *et al.* (2010) and Azawi and Hussein (2013) who reported that inclusion of Vitamin C to Awassi ram and bovine semen extender significantly increased motility at the different times of preservation at 5°C while semen extenders without Vitamin C (control) had lower motile sperm cells respectively. Studies have shown that sperm cells are usually exposed to oxygen and visible light radiation during the process at cryopreservation leading to the formation of reactive oxidative species also known as free radicals (Aysun, 2009) such as Hydroxyl ion, super oxide, lipid peroxides, single oxygen and excess of ROS (reactive oxidative species) impairs motility and capacity of fertilization due to the oxidative stress damage incurred on the sperm cells by free radicals (Bucak *et al.*, 2010). Anti-oxidants such as Vitamin C are agents that break the oxidative chain reaction thereby reducing oxidative stress which leads to the decrease in motility (Bansal and Bilaspuri, 2009). In general anti-oxidants dispose, scavenge and suppress the formation of ROS (Free radicals) hence maintaining motility during cryopreservation (Sikka, 2001).

There was a general though not significant effect on the viability of the sperm cell in the two egg yolk extenders with the three inclusion levels of Vitamin C during the study. After 72 hours, quail egg yolk extender with 3 and 6mg/ml of Vitamin C had significantly higher percentage of viable cells followed by chicken with 3 and 6mg/ml

of Vitamin C with both controls having significantly lower percentage of viable sperm cells. This result agrees with reports by Beconi *et al.*, (1993), Hu *et al.*, (2010) and Asadpour *et al.*, (2011) which showed that Vitamin C supplementation during cryopreservation of bull and ram semen had significant effect on the percentage of viable cells improving percentage viability of sperm cell after preservation.

Sperm cells have a high content of unsaturated fatty acids in their membranes, but lack significant cytoplasmic component containing antioxidants (Sinha *et al.*, 1996). Lipid peroxides are spontaneously generated in the sperm plasma membrane and are released by the action of phospholipase A2 (Bansal and Bilaspuri, 2007). They are capable of inducing DNA damage, decrease in percentage of viable sperm cells and fertility during and after cryopreservation (Bucak *et al.*, 2010). The addition of Vitamin C in extenders can bring about an optimal sperm performance by increasing the percentage of viable cells after cryopreservation by scavenging this lipid peroxides (Hu *et al.*, 2010).

The observation that inclusion of different levels of Vitamin C in chicken and quail egg yolk extenders had no significant effect on pH agrees with Fazeli *et al.*, (2010) who reported that Vitamin C had no significant effect on Markhoz goats seminal pH when added to a semen extender during cryopreservation. Generally pH whether in fresh semen or semen extender ranges from 6 - 7. However, report by Gernah, (2007) showed that dietary intake of Vitamin C by man had significant effect on the seminal pH when assessed.

The non-significant effect of egg yolk type on MPD (Mid piece droplet), DH (Detached head), FT (Free tail), CT (Coiled tail) and BT (Bent tail) of the spermatozoa disagrees with the study of Al-Daraji, (2002) who showed that increase in orange juice as a source of Vitamin C in liquid storage of roosters semen had significantly lower abnormalities

(Head, Neck, Tail) than the control group and also agrees with Hu *et al.* (2010) who reported that increase in supplementation level of Vitamin C up to 6mg/ml decrease the total spermatozoa abnormalities with more normal cells after cryopreservation of bull semen.

It has been shown that mammalian spermatozoa membrane is rich in poly unsaturated fatty acids (PUFAS) which are very sensitive to oxygen induced damage by lipid peroxidation (Sikka, 2001) making them sensitive to reactive oxygen specie which attack the membrane (Desai *et al.*, 2009). Vitamin C as an anti-oxidant, scavenges for the lipid peroxides hence reducing the increasing the percentage of normal cells and also rate of fertility after storage (Al- Daraji, 2002), as shown in the current study.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

There was a general decline in sperm motility and viability across treatments (egg yolk extenders), with increase in storage period ranging from 88 to 66% in chicken egg yolk extender and 82 to 46% in turkey egg yolk extender. However, sperm motility was significantly ($P<0.05$) higher in the chicken and quail egg yolk extenders than other extenders after dilution at first day of storage.

There was significant ($P<0.05$) decline in percentage viable sperms across treatments with increase in storage period. After 72 hours of storage, chicken egg yolk extender was superior (having 71% viable sperms) to other extenders in preserving sperm followed by quail egg yolk extender.

There was a general but no significant ($P>0.05$) decline in percentage of normal sperm cells across treatments with increase in storage period, ranging from 86 to 81% in QEYE and TEYE, 85 to 78% in GEYE and 84 to 82% in CEYE. However, across treatments, there was slightly but no significant ($P>0.05$) difference in percentage normal cells with increase in storage time except in GEYE which had significantly ($P<0.05$) low percentage sperm cells after 72 hours storage than in semen stored in other extenders.

There was a significant ($P<0.05$) increase in percentage sperm abnormalities with increase in length of storage. Across treatments (egg yolk types), DH was slightly higher in semen samples stored in QEYE and TEYE, higher in CEYE and GEYE and higher in TEYE after 24, 48 and 72 hours of storage respectively.

There was significant ($P < 0.05$) with increase in sperm motility with increase in the level of Vitamin C in the extenders over storage periods. However, there was a general, though not significant ($P > 0.05$) increase in percentage of viable spermatozoa with increase in the levels of Vitamin C across the period in all extenders, with chicken egg yolk having the highest number of viable sperms than quail egg yolk extender.

There was generally no significant ($P > 0.05$) decline in percentage of normal sperm cells with increase in vitamin C levels in both chicken and quail egg yolk extenders across storage periods. However, QEYE with 3mg/ml vitamin C had significantly highest percentage of viable cells (88%) and CEYE with 3 and 6mg/ml vitamin C had significantly ($P < 0.05$) the least viable sperm cell.

After 72 hours, there was a significant ($P < 0.05$) decline in percentage of normal sperm cells in all the extenders ranging between 74-84% with CEYE and QEYE having 6mg/ml vitamin C with significantly ($P < 0.05$) the highest percentage of normal sperm cells (84%)

There was a significant ($P < 0.05$) increase in percentage of abnormalities with increase in storage time but there was no significant effect ($P > 0.05$) in percentage of abnormalities with increase in levels of Vitamin C.

6.2 Conclusion

From the result of this study, it can be concluded that chicken egg yolk extender did best in cryopreservation of bull semen and quail egg yolk can be used as an alternative to conventional use of chicken egg yolk due to lower abnormalities of sperm cells stored in it after 72 hours. Inclusion of Vitamin C up to 6mg/ml in both chicken and quail egg yolk semen extenders maintained semen quality by protecting spermatozoa

against harmful effect of lipid peroxidation by free radicals during liquid storage of bull semen up to 72 hours.

6.3 Recommendation

During preparation of semen extender, other avian egg yolks like quail may be used as an alternative to the conventional chicken egg yolk. Also addition of Vitamin C up to 6mg/ml as a major constituent in preparation of semen for storage may be widely adopted as the studies have shown that it helps to maintain the quality of semen during preservation.

REFERENCES

- Adebambo, O. A., Williams, J. L., and Babara, U. (1998). Genetic Variations in Nigerian Cattle Breeds using 26 Micro Satellite Markers. In: Proceedings of the Silver Anniversary Conference of Nigerian Society of Animal Production, Nov. 21-26, 1998, Nigeria, pp. 466-467.
- Agarwal, E., Makker, K., and Sharma, R. (2008). Clinical Relevance of Oxidative Stress in Male Factor Infertility: An Update. *American Journal of Reproductive Immunology*, 59(1): 2–11.
- Agarwal, A., Gupta, S., and Sharma, R.K. (2005). Role of Oxidative Stress in Female Reproductive. *Reproductive Biology and endocrinology*, 28(3): 34-56.
- Agarwal, A. and. Prabakaran, S.A. (2005). Mechanism, Measurement, and Prevention of Oxidative Stress in Male Reproductive Physiology. *Indian Journal of Experimental Biology*, 43(11): 963-974.
- Agarwal, A., Nallella, K. P., Allamaneni, S.S.R., and Said, T.M. (2004). Role of Antioxidants in Treatment of Male Infertility: An Overview of the Literature. *Reproductive Biomedicine Online*, 8(6): 616-627.
- Aires, V.A., Hinsch, K. D., Mueller-Schloesser. F., Bogner, K., Mueller-Schloesser, S., and Hinsch, E. (2003). In vitro and In vivo Comparison of Egg Yolk-based and Soybean Lecithin-based Extenders for Cryopreservation of Bovine Semen. *Theriogenology*, 60(2):69-79.
- Akhter, S., Rakha, B. A., Andrabi, S. M. H., and Ansari, M. S. (2010). Comparison of Egg yolks from Three Avian Species in Extender for Cryopreservation of Sahiwal Bull epididymal spermatozoa. *Animal Science Papers and Reports*, 29(2): 131-138.
- Akpa, G. N., Umar, M. L., and Alphonsus, C. (2011). Evaluation of Fertility and Calving Ease of Small Holder Indigenous Cattle Herds In Semi- Arid Zone of Nigeria. *Iranian Journal of Applied Animal Science*, 1(14): 235-243.
- Akpa, G. N., Alphonsus, C., Duru, S., and Abdulrashid, M. (2006). Factors Affecting Body Size and Horn Length in Small Holder Animals. *Savannah Journal of Agriculture*, 1(2):130-137.
- Al-Daraji, H.J. (2012). Effect of Diluent Supplementation with Different Levels of Orange Juice on Semen Quality during Liquid Storage of Roosters' Semen. *International Journal of Veterinary Science*, 1(1): 5-9.
- Amirat, L., Tainturier, D., Jeanneau, L., Thorin, C., Gérard, O., Courtens, J. L., and Anton, M. (2004). Bull Semen In-Vitro Fertility After Cryopreservation Using Egg Yolk LDL: A Comparison With Optidyl®, A Commercial Egg Yolk Extender, *Theriogenology*, 61:895-907.

- Anton. M. (2007).Low-density lipoproteins (LDL) or Lipovitellenin Fraction. In: Huopalahti, R., López-Fandiño, R., Anton M, Schade R. *Bioactive egg compounds*. Berlin/Heidelberg, Springer-Verlag. pp. 7-12.
- Anton, M., Nau, F., and Nys, Y. (2006).Bioactive egg components and their potential uses. *World's Poultry Science Journal*, 62(4):29-38.
- Asadpour, R., Jafari, R., and Nasrabadi, H. T. (2011).Influence of Added Vitamin C and Vitamin E on Frozen-Thawed Bovine Sperm Cryopreserved in Citrate and Tris-Based Extenders.*Veterinary Research Forum*, 2(1): 37 – 44.
- Aysun, H. (2009). An overview of Ascorbic Acid Biochemistry.*Journal of Faculty of Pharmacy*, 38(3): 233-235.
- Azawi, O. S. and Hussein, E. K. (2013). Effect of Vitamin C or E Supplementation to Tris Diluent on Semen Quality of Awassi Rams Preserved at 5⁰C. *Veterinary Research Forum*, 4(3): 157-160.
- Bailey, J. L., Bilodeau. J. F., and Cormier. N. (2000). Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. *Journal of Andrology*, 21(8): 1-7.
- Ball, B. A., Medina, V., Gravance, C. G., and Baumber, J. (2001). Effects of Oxidants on Preservation of Motility, Viability and Acrosome Integrity of Equine Spermatozoa during Storage At 5⁰C. *Theriogenology*, 56: 577-587.
- Bansal, A.K. and Bilaspuri, G.S.(2007).Effect of Ferrous Ascorbate on In Vitro Capacitation and Acrosome Reaction in Cattle Bull Spermatozoa.*Animal ScienceReport*, 1(2):69-77.
- Bansal, A.K. and Bilaspuri, G.S. (2008^a).Effect of ferrous sulphate and ascorbic acid on motility, viability and lipid peroxidation of crossbred cattle bull spermatozoa. *Animal Reproduction*, 2(1):100-104.
- Bansal, A.K. and Bilaspuri, G.S. (2008^c).Effect of Manganese on Bovine Sperm Motility, Viability and Lipid peroxidation in vitro.*Animal Reproduction CBRA*, 5(4):90-96.
- Bansal, A.K. and Bilaspuri, G.S. (2008^d). Mn²⁺: A Potent Antioxidant and Stimulator of Sperm Capacitation and Acrosome Reaction in Crossbred Cattle Bulls. *Archiv fur Tierzucht*, 51(2):149-156.
- Bansal, A.K. and Bilaspuri, G.S. (2008^b). Oxidative Stress alters Membrane Sulfhydryl status, Lipid and Phospholipid Contents of Crossbred Cattle Bull Spermatozoa. *Animal Reproduction Science*, 104(4):398-404.
- Bansal, A.K. And Bilaspuri, G.S.(2009).Antioxidant Effect of Vitamin E on Motility, Viability and Lipid Peroxidation of Cattle Spermatozoa under Oxidative Stress.*Animal Science Papers and Reports*, 27(1):5-14.
- Bathgate, R., Maxwell, W. M.C., and Evans, G. (2006). Studies on the Effect Of Supplementing Boar Semen Cryopreservation Media With Different Avian Egg

- Yolk Types on In-Vitro Post-Thaw Sperm Quality. *Reproduction of Domestic Animals*, 41: 68-73.
- Barje, P. P. (2006). Utilization of Whole Cottonseed in Diets of Friesian × Bunaji and Bunaji Heifers. Ph. D. Dissertation Submitted to the Department of Animal Science, Ahmadu Bello University, Zaria. Pp 10-58.
- Baumber, J., Ball, B.A. and Gravance, C. G. (2000). The Effect of Reactive Oxygen Species on Equine Sperm Motility, Viability, Acrosomal Integrity, Mitochondrial Membrane Potential and Membrane Lipid Peroxidation. *Journal of Andrology*, 21: 895-902.
- Bearden, H. J. and Fuquay, J. W. (2000). *Applied Animal Reproduction*. 5th Edition. Upper Saddle: New Jersey: Prentice hall Inc ; Pp138-147.
- Beconi, M. T., Francia, C. R., and Mora, N. G. (1993). Effect of Natural Antioxidants in Frozen Bovine Semen Preservation. *Theriogenology*, 40: 841-851.
- Bergeron, A., Brindle, Y., Blondin, P. and Manjunath, P. (2007). Milk Caseins Decrease the Binding of the Major Bovine Seminal Plasma Proteins to Sperm and Prevent Lipid Loss from the Sperm Membrane During Sperm Storage. *Biology of Reproduction*, 77(1): 20-61.
- Blench, R. (1999). Traditional Livestock Breeds: Geographical Distribution and Dynamics in Relation to the Ecology of West Africa. Overseas Development Institute Portland House Stag Place. London. Pp. 14-21.
- Blench, R. (1993). Trends in the Distribution of Traditional Livestock Breeds in Nigeria and Explanatory Models. In: *Animal Genetics and Health*. Rome: Food and Agriculture Organisation of United Nations.
- Blench, R. M., Maitland, P., De Jode, A., Gherzi, E., Di Dominico, C., Hall, S., and Sambo, N. A. (1998). West African Dwarf Shorthorn Cattle in Nigeria: History, Distribution and Productivity. In: Seignobos, C and Thys, E. Eds. *Des Taurins au Cameroun et Nigeria*. 249-292. Paris: ORSTOM/IEMVT, Maisons-Alfort.
- Blezinger, S. (1999). Cattle Today: Many Factors Affect Bull Performance. <http://www.cattletoday.com>
- Brito, L. F. C., Silva, A. E. D. F., Rodrigues, L. H., Vieira, F. V., Deragon, L. A. G., and Kastelic, J. P. (2002): Effect of Environmental Factors, Age and Genotype on Sperm Production and Semen Quality in *Bos indicus* and *Bos taurus* in Brazil. *Animal Reproduction Science*. 70, 181-190.
- Bucak, M.N., Tessahin, A.A., Varisl, O., Yuce, A., Tekin, N., and Akcay, A. (2007). The Influence of Trehalose, Taurine, Cysteamine and Hyaluronan on Ram Semen. Microscopic and Oxidative Stress Parameters after Freeze-thawing Process. *Theriogenology*. 67(5):1060-1067.
- Bucak, M. N., Tuncer, P.N., Sariozkan, S., and Ulutas, P. A. (2010). Comparison of the Effects of Glutamine and an Amino Acid Solution on Post-thawed Ram Sperm

- Parameters, Lipid Peroxidation and Anti-oxidant Activities.*Small Ruminant Research*, 81(1):13-17.
- Bucak, M.N., Atessahin, A., and Yuce, A. (2008).Effect of Anti-oxidants Antioxidative Stress Parameters on Ram Semen after the Freeze-thawing Process.*Small Ruminant Research*, 75(3):128-134.
- Burris, C. And Webb,G.(2009).Effects of Egg Yolk Source on the Cryopreservation of Stallion Semen.*Journal of Equine Veterinary Science*, 29(33): 6-7.
- Butswat, I. S. R., Kalla, D. J. U., Mbap, S. T., Moloku, J. U., and Chuo, P. B. (2001). Evaluation of Reproduction Problems of Friesian, Bunaji and Crossed Cows in Subtropical Environment. *Nigerian Journal of Animal Production*, 28(2): 123-127.
- Celeghini, E.C.C., De Arruda, R.P., De Andrade, A.F.C., Nascimento, J., Raphael, C.F., and Rodrigues, P.H.M. (2008).Effects that Bovine Sperm Cryopreservation Using Two Different Extenders have on Sperm Membranes and Chromatin. *Animal Reproduction Science*, 104: 119-131.
- Chacon, J., Pere, Z. E., Mueller, E., Soderquist, L., and Rodríguez-Martinez, H. (1999).Breeding soundness examination of extensively managed bulls in Costa Rica.*Theriogenology*, 52: 21-231.
- Chatterjee, S., De Lamirande, E., and Gagnon, C. (2001). Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. *Molecular Reproduction and Development*, 60(4):498-506.
- Cheema, R.S., Bansal, A.K., and Bilaspuri, G.S. (2009).Manganese provides antioxidant protection for sperm cryopreservation that may offer new consideration for clinical fertility. *Oxidative Medicine and Cellular Longevity*, 2(3):152-159.
- Chenoweth. P. J. (2005).Genetic Sperm Defects.*Theriogenology*, 64: (4)57-68.
- Clulow, J. R., Maxwell, W. M. C., Evans, G., and Morris, L. H. A. (2007).A Comparison of Duck and Chicken Egg Yolk for Cryopreservation of Stallion Sperm.*Australian Veterinary Journal*, 85(23): 2-5.
- DeJarnette, J. M. (2005). The Effect of Semen Quality on Reproductive Efficiency.*Veterinary Clinic North Food AnimalPractice*, 21(40): 9-18.
- Desai, N.,Sharma, R., Maker, K., Sabnegh, E., and Agarwal, A. (2009). Physiological and Pathological Levels of Reactive Oxygen Species in Neat Semen of Infertile Men.*Fertility and Sterility*, 92: 1626–1631.
- Dorado, J., Serrano, A.M. and Hidalgo, M. (2010). The Effect of Cryopreservation on Goat Semen Characteristics Related to Sperm Freezability. *Journal of Reproductive Science*, 12(1): 115-123.
- Du Plessis S. S., Makker,K. N., Desai, R., and Agarwal, A. (2008).Impact of Oxidative Stress on IVF.*Expert Review of Obstetrics and Gynaecology*, 3(4): 539–554.

- Duncan, D. B. (1955). Multiple Ranges and Multiples F-tests. *Biometrics*, 11: 1-42.
- Eddy, E. M. (2006). The Spermatozoon. In: Wassarman PM, Neill JD, editors. Knobil and Neill's physiology of reproduction, 3rd ed. St Louis: Academic Press, pp 3-54.
- Engelman, D. M. (2005). Membranes are More Mosaic than Fluid. *Nature*, 438(5): 78-80.
- Fafchamps, M. and Gavian, S. (1997). The Determinants of Livestock Prices in Niger. *Journal of African Economies* 6(2):255–295.
- FAO(2011). Food and Agriculture Organization Statistics of Live Animals. <http://faostat.fao.org/site/573/DesktopDefault.aspx?PageID=573>, September.
- Fazeli, P., Zamiri, M. J., Farshad, A., and Khalili, B. (2010). Effects of Vitamin C on Testicular and Seminal Characteristics of Markhoz Goats. *Iranian Journal of Veterinary Research*, 11(3): 1-6.
- Felius, M. (1995). Cattle Breeds: An Encyclopaedia. Misset, Doetinchem. pp. 799. The Netherlands.
- Flesch, F.M. and Gadella, B.M. (2000). Dynamics of the Mammalian Sperm Plasma Membrane in the Process of Fertilization. *Biochemistry*, 146(9): 197-235.
- Foote, R. H. (2002) The History of Artificial Insemination: Selected Notes and Notables. *Journal of Animal Science*, 80:1-10.
- Foote, R. H., Brockett, C. C. and Kaproth, M. T., (2002). Motility and Fertility of Bull Spermatozoa in Whole Milk Extender Containing Antioxidants. *Animal Reproduction Science*, 66(4): 430-434.
- Ford, W.C. (2001), Reactive oxygen species and sperm. *Human fertility*, 4: 77-78.
- Frydrychová, S., Čerňanský, J., Lustyková, A., and Rozkot, M. (2010). Effects of Long-Term Liquid Commercial Semen Extender and Storage Time on the Membrane Quality of Boar Semen. *Czech Journal of Animal Science*, 55: 160-166.
- Fuerst-Waltl, B., Schwarzenbacher, H., Perner, C. and Solkner, J. (2006). Effects of Age and Environmental Factors on Semen Production and Semen Quality of Austrian Simmental Bulls. *Animal Reproduction Science*, 95(1): 27-37.
- Gadea, J., Vasquez, F.G., Matas C., Gardon, J. C., Canovas. S., and Gumbao D. (2005). Cooling and Freezing Boar Spermatozoa: Supplementation of the Freezing Media with Reduces Glutathione Preserves Sperm Function. *Journal of Andrology*. 6(9):3.
- Gadella, B. M., Rathi, R., Brouwers, J. M., Stout, T. A. E., and Colenbrander, B. (2001). Capacitation and the Acrosome Reaction in Equine Sperm. *Animal Reproductive Science*, 68(2): 49-65.

- Garrido, N., Meseguer, M., and Simon, C. (2004). Proxidative and Antioxidative Imbalance in Human Semen and its Relation with Male Infertility. *Asian Journal of Andrology*, 6(1): 59-65.
- Gates, G. M. (1992). Livestock and Land- use in Sub-Saharan Africa. *Oxfam Research Paper*, Oxfam (UK and Ireland), Oxford. Pp 11.
- Gernah, D. I., Atolagbe, M.O., and Echegwo, C.C. (2007). Nutritional Composition of the African Locust Bean (*Parkia Biglobosa*) Fruit Pulp. *Nigerian Food Journal*, 25 (1):190-196.
- Gholami, M., Faraji, Z., and Zamiri, M. J. (2012). Effect of Egg Yolk of Four Avian Species on the Cryopreserved Ram Spermatozoa. *Iranian Journal of Veterinary Research*, 13(1):1-5.
- Gordon, I. (2004). Artificial Insemination. In: Gordon, I. (Ed) *Reproductive Technologies in Farm Animals*. Oxfordshire: CABI Publishing, pp 49-81.
- Graham, J.K. and Mocé.E. (2005) Fertility Evaluation of Frozen-thawed Semen. *Theriogenology*, 64: 492-504.
- Gravance, C. G., Casey, M. E. and Casey, P. J. (2009). Pre-freeze Bull Sperm Head Morphometry Related to Post-Thaw Fertility. *Animal Reproduction Science*, 114: 81-88.
- Hafez, E. S. E. (1993). Reproduction in Farm Animals. 6th Ed. Wiley-Blackwell' Philadelphia, PA, pp. 405-439.
- Hafez, B. (2000). Reproduction in Farm Animals. 7th Ed. Wiley-Blackwell, Philadelphia, PA, USA.
- Hall, S. J. G. (1991). Body Dimensions of Nigerian Cattle, Sheep and Goats. *Animal Production*, 53(3): 61-69.
- Hancock, J. L. (1957). A Staining Technic for Study of Temperature Shock in Semen. *Nature*. London. 197: pp 323- 343.
- He, L., Bailey, J. L., and Buhr, M. M. (2001). Incorporating Lipids into Boar Sperm Decreases Chilling Sensitivity but not Capacitation Potential. *Biology of Reproduction*, 64:69-79.
- Hoffmann, I. (2010). Climate Change and the Characterization, Breeding and Conservation of Animal Genetic Resources. *Animal Genetics*, 41(1):32-46.
- Hu, J., Tan, W., and Zhoa X. (2010). The Cryoprotective Effect of Ascorbic Acid Supplementation on Bovine semen Quality. *Animal Reproductive Science*, 12(1):71-72.
- Hunderra, S. A. (2004). Evaluation of Semen Parameters in Ethiopian Indigenous Bull kept at Kaliti Artificial Insemination Center, Addis Ababa.

- Ibrahim, E., El-Shenawy, E., Ahmed, H., Mohammed, E., and Mohamed, E. (2012). A Comparison of Duck And Chicken Egg Yolk for Cryopreservation Of Egyptian Buffalo Bull Spermatozoa. *Animal Reproduction Science*, 40: 1-6.
- ILCA. (1991). Working Document No. 15. A Handbook for African Livestock Statistics ILCA, Addis Ababa, Ethiopia. Mitcher, A. E. and Tuffs. R. J. 1989. Humans Books Inc. Edmonton, Canada. Pp 145.
- Jimenez-Severiano, H., Quintal-Franco, Vega-Murillo, V., Zanella, E., Lindsey, R. and Kinder, J. E. (2003). Season of the Year Influences Testosterone Secretion in Bulls Administered Lutenizing Hormone. *Journal of Animal Science*, 81: 1023-1029.
- Karolina, B. (2012). Bull Semen Collection and Analysis for Artificial Insemination. *Journal of Agricultural Science*, 4(3): 127-133.
- Kohler-Rollefson, I., Rathore, H. S., and Mathias, E. (2009). Local Breeds, Livelihoods and Livestock Keepers' Rights in South Asia. *Tropical Animal Health Production*, 41(7): 1061-1070.
- Kumar, H. and Mahmood, S. (2001). The Use of Fast acting Antioxidants for the Reduction of Cow Placental Retention and Subsequent Endometritis. *Indian Journal of Animal Science*, 71(7): 650-653.
- Kulaksız, R., Çebi, C., Akçay, E., and Daşkın, A. (2010). The Protective Effect of Egg Yolk from Different Avian Species during the Cryopreservation of Karayaka Ram Semen. *Small Ruminant Research*, 88(1): 2-5.
- Lewis, S.E.M., Sterling, E. S. L., and Young, I. S. (1997). Comparison of Individual Antioxidants of Sperm and Seminal Plasma in Fertile and Infertile Men. *Fertility and Sterility*, 67: 142-147.
- Lunde, H. and Lindtjorn, G. (2013). Cattle and Climate in Africa, How Climate Variability has Influenced National Cattle Holdings from 1961-2008. doi: 10.77171/Peerj55.
- MacDonald, K. C. and MacDonald, R. H. (1999). The Origins and Development of Domesticated Animals in Arid West Africa. In: Blench R. M and MacDonald. K. C (Ed). *The origin and development of African livestock*. University College Press, London, England, pp 23-46.
- Maldjian, A., Pizzi, F., Gliozzi, T., Cerolini, S., Penny, P., and Noble, R. (2005). Changes in Sperm Quality and Lipid Composition During Cryopreservation of Boar Semen. *Theriogenology*, 6 (4): 11-21.
- Mandal, D. K., Nagpaul, P. K., and Gupta, A. K. (2002). Effects of Body Surface Cooling During Hot Dry and Hot Humid Seasons on Seminal Attributes of Murrah Buffalo Bulls. *Indian Journal of Animal Sciences*, 72 (2): 192-194.
- Manjunath P., Nauc V., Bergeron A., and Menard M. (2002). Major Proteins of Bovine Seminal Plasma Bind to the Low-Density Lipoproteins Fraction of Hen's Eggs Yolk. *Biology of Reproduction*, 67: 1250-1258.

- Mathevon, M., Buhr, M. M., and Dekker, J. C. M. (1998). Environmental Management and Genetic Factors Affecting Semen Production in Holstein Bulls. *Journal of Dairy Science*, 81: 3321-3330..
- Mbap, S. T. and Ngere, L. O. (1995). Upgrading of White Fulani Cattle in Vom Using Friesian Bulls. *Tropical Agriculture (Trinidad)*, 72: 152-157.
- Meghen, C., MacHugh, D. E., Sauveroche, B., Kana, G., and Bradley, D. G. (1999). /Characterisation of the Kuri Cattle of Lake Chad using Molecular Genetic Techniques. In: Blench R. M and MacDonald. K. C. (Ed) *The origin and development of African livestock*. University College Press, London, England, pp. 12-15.
- Meyers, S. A. (2006). Sperm Physiology. In: Samper JC, Editor. *Equine Breeding Management and Artificial insemination*, 2nd ed. Saint Louis: W.B. Saunders, pp. 47-55.
- Moussa.M., Martinet, V., Trimeche.A., and Tainturier, D., and Anton, M. (2002). Low Density Lipoproteins Extracted from Hen Egg Yolk by an Easy Method: Cryoprotective Effect on Frozen-thawed Bull Semen. *Theriogenology*, 57(16): 95-706.
- Nagy, S., Jansen, J., Topper, E. K., and Gadella, B. M. (2003). A triple-stain Flow Cytometric method to Assess Plasma and Acrosome Membrane Integrity of Cryopreserved Bovine Sperm immediately after Thawing in Presence of Egg-yolk particles. *Biology of Reproduction*, 68(18): 28-35.
- Ngere, L. O. (1990). *Endangered Livestock Breeds in West Africa*. *Animal Genetic Resources*. A Global Programme for Sustainable Development. FOA Animal Production and Health, Food and Agriculture Organisation of the United Nations, Rome, Italy, Paper 80. Pp. 189-196.
- Noakes, D. E., Parkinson, T. J., and England G. C. W. (2009). *Veterinary Reproduction and Obstetrics*. London: Saunders Elsevier, pp 750-760.
- Nichi, M., Bols, P. E., Zuge, R. M., and Barnabe, V. H. (2006). Seasonal Variation in Semen Quality in *Bos Indicus* and *Bos Tarus* Bulls raised under Tropical Conditions. *Theriogenology*, 66: 822-828.
- Oni, O. O., Adeyinka, I. A., Ajolayan, R. A., Nwagu, B. I., Malan Abdul, A. E. O., Alawa, C. B. I., and Zanobe, O. S. (2001). Relationship between Milk yield, Postpartum Body Weight and Productive Performance in Friesian Bunaji Cattle. *Asian-Australian Journal of Animal Science*, 11(9): 1516-1519.
- Pasqualotto, F.F., Sharma, R.K., Potts, J.M., Nelson, D.R. Thomas Jr., A.J. and Agarwal, A. (2000). Seminal Oxidative Stress in Patients with Chronic Prostatitis. *Urology*, 55(6): 881-885.
- Payne, W. J. A. and Hodges, J. (1997). *Tropical Cattle: Origins, Breeds and Breeding Policies*. Blackwell Science, Oxford, U.K. pp 318.

- Rauch, A. (2013). Cryopreservation of Bovine Semen in Egg Yolk based Extender. M. Sc Dissertation Submitted to the University of Saskatchewan. Pp 120-233.
- Reece, W. O. (2005). Functions Anatomy and Physiology of Domestic animals, 3rd edition. Baltimore, Lippincott, Williams and Wilkins.
- Reddy, N. S. S., Mohanarao, G.J. and Atreja, S.K. (2010). Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (*Bubalus bubalis*) sperm quality following cryopreservation. *Animal Reproduction Science*, 119:183-190.
- Rege, J. E. O. (1999). The State of African Cattle Genetic Resources and Threatened Extinct Breeds. *Animal Genetic Resources Information*. 25(26): 1-25.
- Rege, J. E. O. and Tawah, C. L. (1999). The State of Africa Cattle Genetic Resources II. Geographical Distributions, Characteristics and Uses of Present Day Breeds and Strains. *FOA/UNEP Animal Genetic Resources Information Bulletin*, 26(9): 1-25.
- Rekwot, P. I., Oyedipe, E. O., Dawuda, P.M., and Sekoni, V. O. (1997). Age And Hourly Related Changes Of Serum Testosterone and Spermogram of Pre- Pubertal Bulls Fed Two Levels of Nutrition. *The Veterinary Journal*, 153: 341-347.
- Rekwot, P. I., Lamidi, O. S., Adamu, A. M., Egbuedo, C. U., Ruwaan, J. S. and Okereke, S. N. (1987). Reproductive Performance of Bunaji Bulls Grazing Natural Pasture and Receiving Supplement Containing Palm Kernel Meal. *Nigerian Veterinary Journal*, 18: 26-36.
- Ricker, J. V., Linfor, J. J., Delfino, W. J., Kysar, P., Scholtz., E. L., and Tablin, F. (2006). Equine Sperm Membrane Phase Behaviour: The Effects of Lipid-based Cryoprotectants. *Biology of Reproduction*, 74(3): 59-65.
- Rowen, D. F., Wilke W.L., and Fails, A. D. (2011). Anatomy and Physiology of Farm Animals 7th Edition. Wiley-Blackwell, A John Wiley and Sons, Inc, Publication.
- Saacke, R. G., Dalton, J.C., Nadir, S., Nebel, R. L., Dejarnette, J. M., and Bame. J. H. (1994). Assessing Sperm Evaluation and Bull Fertility. *An Update Proceedings of the 15th Technical Conference on Artificial Insemination and Reproduction NAAB*. pp 57-67.
- Saacke, R. G., Dalton, J.C., Nadir, S., Nebel, R. L., and Bame. J. H. (2000). Relationship of Seminal Traits and Insemination Time to Fertilization Rate and Embryo Quality. *Animal Reproduction Science*, 66: 60-61.
- Said, T.M., Grunewald, S., Paasch, U., Agarwal, A., and Glander, H.J. (2005). Effects of Magnetic-activated Cell Sorting on Sperm Motility and Cryosurvival Rate. *Fertility and Sterility*, 83(5):1442-1446.
- Sanocka, D. and Kurpysz, M. (2004). Reactive Oxygen Species and Sperm Cells. *Reproductive Biology and Endocrinology*, 2: 12-26.
- Sariozkan, S., Bucak, M.N., Tuncer, P.B., Ulutas, P.A., and Bilgen, A. (2009). The Influence of Cysteine and Taurine on Microscopic-Oxidative Stress Parameters

- and Fertilizing ability of Bull Semen Following Cryopreservation. *Cryobiology*, 58(2): 134-138.
- Seo, S. and Mendelsohn, R. (2006). The Impact of Climate Change on Livestock Management in Africa: A Structural Ricardian Analysis. CEEPA Discussion Paper, 23:1–48.
- Sikka, S.C. (2001). Relative Impact of Oxidative Stress on Male Reproductive Function. *Current Medicinal Chemistry*, 8(7): 851-862.
- Silva, P. F. N. (2006). Physiology of Peroxidation process in Mammalia Spermatozoa. Phd Thesis, Utrech University, Ripperprint, Ridderk. Pp 5-36.
- Singh, A., Veers, U., and Singh, S. (2012). Semen Evaluation in Farm Animals. *International Journal of Biomedical and Life Science*, 2(1): 35-42.
- Sinha, M. P., Sinha, A. K., and Singh B. K. (1996). The Effect of Glutathione on the Motility, Enzyme Leakage and Fertility of Frozen Goat Semen. *Theriogenology*, 41: 237-243.
- Sorensen Jr, A. M. (1997). Reproduction Laboratory. A laboratory Manual for Animal Production. 3rd Edition. Kent all/Hunt Publishing Company, Iowa, U.S.A. pp151.
- Sri, W., Hermanto, N., Agus, B., and Panji, B. (2012). Effects of Sperm Concentration and Length of Storage at 5⁰C on Motility of Goat Spermatozoa. *World Academy of Science, Engineering and Technology*, 66:1099-1101.
- Statistical Analysis System (2002). SAS User's Guide. SAS institute. Inc. Cary NC.
- Strzezka, J. (2007). Biological Condition of the Male Reproductive Valve. *Biological Reproduction of Animal*, 2(7): 133-157.
- Su, L., Li, X., Quan, J., Yang, S., Li, Y., and He, X. (2008). A Comparison of the Protective Action of Added Egg Yolks from Five Avian Species to the Cryopreservation of Bull Sperm. *Animal Reproduction Science*, 104 (21): 2-9.
- Suarez, S. S. (2008). Control of Hyperactivation in Sperm. *Human Reproductive Update*, 14(6): 47-57.
- Sugulle, A. H., Bhuiyan, M. U., and Shamsuddin, M. (2006). Breeding Soundness of Bulls and Quality of their Frozen Semen used in Cattle Artificial Insemination in Bangladesh. *Livestock Research for Rural Development*, 18(54): 14-18.
- Taddesse, G., Peden, D., Abiye, A., and Wagnaw A. (2003). Effect of Manure on Grazing Lands in Ethiopia, East African Highlands. *Mountain Research and Development*, 23(2):156–160.
- Taylor, J. F., Bean, B., Marshall, C. E., and Sullivan, J. J. (2002). Genetic and Environmental Components of Semen Production Traits of Artificial Insemination of Holstein Bulls. *Journal of Dairy Science*, 123: 27-38.

- Terblanche, J. S., Clusella-Trullas, S., Deere, J. A., and Chown Steven, L. (2008). Thermal Tolerance in a South-east African Population of the Tsetse Fly *Glossina Pallidipes* (diptera, glossinidae). Implications for Forecasting Climate Change Impacts. *Journal of Insect Physiology*, 54(1):114–127.
- Thiebier, M. and Guerin, B. (2000). Hygienic Aspects of Storage and Use of Semen for Artificial Insemination. *Animal Reproductive Science*, 62(2): 33-51.
- Thiebier, M., and Wagner, H.G. (2002). World Statistics for Artificial Insemination in Cattle. *Livestock Production Science*, 74(20): 3-12.
- Trimeche, A., Anton, M., Renard, P., Gandemer, G., and Tainturier, D. (1997). Quail Egg yolk: A Novel Cryoprotectant for the Freeze Preservation of Poitou Jackass Sperm. *Cryobiology*, 34(3): 85- 93.
- Uysal, O. and Bucak, M. N. (2007). Effects of Oxidized Glutathione, Bovine serum Albumin, Cysteine and Lycopene on the Quality of Frozen-thawed Ram Semen. *Acta Veterinaria Brno*, 76(3):383-390.
- Van Wagendonk-de Leeuw, A. M., Haring, R. M., Kaal-Lansbergen, L. M., and Den Daas J. H. (2000). Fertility Results using Bovine Semen Cryopreserved with Extenders based on Egg Yolk and Soy Bean Extract. *Theriogenology*, 54:57-67.
- Violeta, I., Moje, A., Mircu, C., Roman, C., and Delia, C. (2010). The Influence of Some Environmental Factors and Age on Semen Production of Fleckvieh Bulls. *Scientific Journal of Veterinary Medicine*, 119(2): 56-63.
- Vishwanath, R. (2003). Artificial insemination: The State of the Art. *Theriogenology*, 59(5): 71-84.
- Vishwanath, R. and Shannon, P. (2000). Storage of Bovine Semen in Liquid and Frozen State. *Animal Reproduction Science*, 62: 23-53.
- Watson, P.F. (2000^a). The Protection of Ram and Bull Spermatozoa by Low Density Lipoprotein Fraction of Egg Yolk during Storage at 5^oC and Deep Freezing. *Journal of Thermal Biology*, 137(1): 41-50.
- Watson, P.F. (2000^b). The Causes of Reduced Fertility with Cryopreserved Semen. *Animal Reproduction Science*, 61(4): 81–92.
- Wierzbowski, S. and Zukowski, K. (2007). Cattle Reproduction. Balice KOS. pp 127-133.
- Wint, W. and Robinson, T. (2007). Gridded Livestock of the World. Technical Report, Food and Agriculture Organization of the United Nations, Rome. pp 27-45
- Yamauchi, S., Nakamura, S., Lay, K. M., Azuma, T., Yakabi, T., and Muto, N. (2009). Characteristics of Okinawan Native Agu Pig Spermatozoa after Addition of Low-Density Lipoprotein to Freezing Extender. *Journal of Reproductive Development*, 55(5): 58-65.

- Yousef, M.I., Abdallah, G.A., and Kamel, K.I. (2003). Effect of Ascorbic Acid and Vitamin Supplementation on Semen Quality and Biochemical Parameters of Male Rabbits,” *Animal Reproduction Science*, 76(1-2):99-111.
- Yue, D., Yan, L. and Luo, H. (2010).Effect of Vitamin E Supplementation on Semen Quality and Testicular Cell Membrane and Mitochondrial Antioxidant Abilities in Aohan Finewool Sheep.*Animal Reproduction Science*, 118: 217-222.
- Zemjanis, R. (1971). Diagonistic and Therapeutic Techniques in Animal Reproduction. 2nd Edition Baltimore, The Williams and Wilkins Co.