EFFECTS OF ASCORBIC ACID AND ALPHA-TOCOPHEROL ON SEMEN CHARACTERISTICS, SERUM TESTOSTERONE AND HAEMATOLOGICAL PARAMETERS OF NIGERIAN MIXED-BREED DOGS IN ZARIA

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SEPTEMBER, 2012

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILMENT FOR THE AWARD OF MASTER OF SCIENCE IN THERIOGENOLOGY

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SEPTEMBER, 2012

DECLARATION

I hereby declare that the research work in this thesis entitled 'Effects of Ascorbic acid and Alpha-tocopherol on Semen Characteristics, Serum Testosterone and Haematological Parameters of Nigerian Mixed-breed Dogs in Zaria' was carried out by me in the Department of Theriogenology and Production, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria under the supervision of Professor E. K. Bawa, Professor J. O. Ayo and Dr. I. U. Ate.

No part of this thesis has been previously presented elsewhere for any higher degree or diploma. The information by other authors cited has been duly acknowledged by references provided.

Rashidah Abimbola MUSTAPHA

| _____ | _____ | _____ |
| **Name of Student** | **Signature** | **Date** |

CERTIFICATION

This thesis titled "Effects of Ascorbic acid and Alpha-tocopherol on Semen Characteristics, Serum Testosterone and Haematological Parameters of Nigerian Mixed-breed Dogs in Zaria" meets the regulation governing the award of degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its scientific contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to the loving memories of my sweet mother, Hajia Misturah Aduke Odeyemi (Alhaja Oluyole). I thank Allaah for coming to this world through you, Hajia, and for your unending encouragement throughout my undergraduate studies. However, you were not there to accompany me to take the long-awaited oath into this noble profession. May The Most Merciful bless and rest your soul. Amen.

ACKNOWLEDGEMENTS

ABSTRACT

The aim of this study was to investigate the effects of antioxidant vitamins Ascorbic acid (AA), vitamin E (VE) and their combination (AA + VE) on semen characteristics, serum testosterone level and haematological parameters. Nine (n = 9) healthy, sexually matured, Nigerian mixed-breed male dogs aged 2-3 years with an average weight of 20 ± 2 kg were used, and were assigned into treated group of five (n = 5) and untreated group of four (n = 4). The experiment was conducted in three phases, each lasting four weeks, with an interval of four weeks between each treatment phase. Dogs (n=5) were orally treated with 5 mg/kg AA (Phase 1), or 5 mg/kg VE (Phase 2), or 5 mg/kg AA + 5 mg/kg VE (Phase 3), while the control (n=4) received 5 ml normal saline for four weeks. Semen was collected once weekly using the penile massage technique, and evaluated for physical characteristics. Blood samples were obtained via the cephalic vein using 5 ml syringe and 21G x $1^{1}/_{2}$ inch needle for haematology, and serum was extracted for determination of testosterone levels. Results were expressed as mean $\pm$ standard error of mean (mean $\pm$ S.E.M). Semen volume was significantly higher ($P < 0.05$) in the AA-treated group ($2.81 \pm$ 0.33 mL) than in the VE-, AA+VE-treated and the control dogs (1.47 ± 0.34 mL, 1.79 ± 0.20 ml and 1.21 ± 0.11 mL, respectively). There was no significant difference ($P > 0.05$) between the VE-treated and the control group, but the AA+VE-treated group and the control group were significantly different ($P < 0.05$). Percentage sperm motility were significantly higher ($P < 0.05$) in the supplemented dogs (AA = 82.50 ± 2.81 %, VE = $86.14 \pm$ 1.90 % and AA+ VE = 86.88 ± 5.28 %) than the control (67.72 ± 2.50 %). There were no significant differences ($p > 0.05$) in the percentage mass motility among the treated groups. The values of semen concentration recorded in all treated groups were significantly higher ($p <$

0.05; AA = 308 8 $\pm$ 32.44 x 10^6/mL, VE = 388.10 $\pm$ 28.42 x 10^6/mL and AA+VE = 445.30 $\pm$ 19.63 x 10^6/mL) than in the control (198.5 $\pm$ 9.67 x 10^6/mL). There were no significant differences (P > 0.05) observed between the VE-treated dogs and the AA+VE-treated dogs. However, the highest semen concentration was recorded in the AA+VE- treated dogs. The AA-treated group recorded the lowest value of percentage live sperm (86.33 $\pm$ 1.86 %) among the treated and control groups, and the obtained value was not significantly different from the control group (P > 0.05; 89.40 $\pm$ 0.85 %), but significantly different from the VE- and AA+VE-treated dogs (P < 0.05; 91.00 $\pm$ 1.07 % and 91.59 $\pm$ .75 %, respectively). The value of overall percentage sperm defects was significantly lower in the VE-treated group (P < 0.05; 2.83 $\pm$ 0.46 %) than in the AA-, AA+VE-treated and the control groups (7.02 $\pm$ 1.31 %, 6.45 $\pm$ 1.13 % and 6.32 $\pm$ 0.79, respectively). The AA-treated group recorded the highest defects among all groups. As treatment progressed, significant differences (P > 0.05) were observed among the mean serum testosterone levels among the AA-, VE-treated dogs and the control. However, there was no significant difference between the AA+VE-treated group and the control. There were significant differences (P < 0.05) between the values of packed cell volume and haemoglobin concentration recorded in the AA+VE-supplemented group (43.67 + 2.62 % and 14.54 $\pm$ 0.54 g/dl, respectively) and those in the AA-, VE-treated and control groups. There were no significant differences (P > 0.05) in total and absolute leukocyte count between the treated dogs and the control. In conclusion, supplementation with the combination of vitamins C and E, or vitamin E alone in the Nigerian mixed-breed dog enhanced semen quality while supplementation with either vitamin C or E increased serum testosterone levels. Supplementation with the combined Vitamins C and E increased the erythrocytic parameters of the Nigerian mixed breed dog.

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LIST OF ABBREVIATIONS

AA   Ascorbic acid

AA+VE   Ascorbic acid and vitamin E

AKC   American Kennel Club

AMD   Age-related macular degeneration

ANOVA   Analysis of variance

BT   Bent tails

CBC   Complete blood count

CT   Coiled tails

DH   Detached heads

DHLPP   Distemper, Hepatitis, Leptospirosis, Parvoviral and Parainfluenza

DHT   5-alpha-dihydrotestosterone

DNA   Deoxyribonucleic acid

FAO   Food and Agriculture Organisation

FSH   Follicle stimulating hormone

FT   Free tails

G6PD   Glucose-6-phosphate dehydrogenase

Hb   Haemoglobin concentration

HDL   High-density lipoprotein cholesterol

LDL   Low-density lipoprotein

LH   Luteinizing hormone

MCH   Mean corpuscular haemoglobin

MCHC   Mean corpuscular haemoglobin concentration

MCV   Mean corpuscular volume

MPD   Mid-piece (cytoplasmic) droplets

mRNAs	messenger Ribonucleic acids
NRCNA	National Research Council of the National Academies
OH	Hydroxyl radical
PCV	Packed cell volume
PUFAs	Polyunsaturated fatty acids
ROO-	Lipid peroxyl radical
ROOH	Lipid hydroperoxides
ROS	Reactive Oxygen Species
SHBG	Sex hormone-binding globulin
SPSS	Statistical Package for Social Sciences
SSCs	Spermatogonial stem cells
EIA	Enzyme immunoassay
USDA	United State Department of Agriculture
VE	Vitamin E
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Dogs are pack animals with a complex set of behaviours related to determining each dog's position in the social hierarchy. They exhibit various postures and other means of non-verbal communication that reveal their states of mind (Dirven and Verspoor, 2004). These sophisticated forms of social cognition and communication may account for their ability to fit into human households and social situations. These attributes have earned dogs a unique relationship with humans despite being potentially dangerous apex predators (Broz, 2008). With the development of modern understanding of genetics, humans began to intentionally breed dogs for a wide range of specific traits. Through this process, the dog has developed into hundreds of varied breeds, and shows more behavioural and morphological variation than any other land mammal (Spady and Ostrander, 2008). Dogs interbreed freely, except where extreme variations in size exist, so mixed-breed dogs vary in size, shape and colour, making them difficult to be classified physically (Ostrander, 2007). Dog has been domesticated for over 15,000 years (Lindblad *et al.,* 2005), and it diverged into a handful of groups of similar animals with functional roles such as earliest role of hunting (Ruusila and Pesonen, 2004), as pets (Nast, 2006), work/service dogs (Franklin, 2006), for sports and shows (American Kennel Club, 2005) and as food source amongst others (Arthur, 2001; Pettid, 2008). The relationship between dog and man has spanned into the economy of man, as breeding of dogs has assumed an increasingly profitable economic venture providing sustainable means of livelihood to breeders in many cities in Nigeria and worldwide (Stanley, 2003).

The mixed-breed dog, derogatively called *mutt*, or *mongrel*, is genetically healthier (Parker *et al.,* 2004), with a higher mean lifespan (Profshosky *et al.,* 2003) and a number of more desirable reproductive traits than the pure breed. It is difficult to trace the ancestry of a mixed-breed dog even by knowledgeable dog observers because mixed-breeds have much more genetic variation than purebreds (Parker *et al.,* 2004).

Sexual maturity begins at the age of six to twelve months for both males and females, although it can be delayed until up to two years old for some large breeds (Feldman and Nelson, 1996). This is the time at which female dogs will have their first oestrus, and the male is ready to copulate because it is attracted to the bitch during oestrus. Domestic male dogs may remain reproductively competent year-round and be ready to mate in the presence of females in estrus (Asa, 1998). However, diseases and seasonal variations have been reported to affect some reproductive traits such as semen quality (Casao *et al.,* 2010), sperm output (Verstegen *et al.,* 2002), and serum testosterone (Amann, 1986). Hence the need to perform a thorough prebreeding examination of stud dogs which involves a complete physical examination, screening for antibodies to *Brucella canis*, semen evaluation, and possibly, a genetic screening test appropriate for a given breed (Hafez and Hafez, 2000).

Testicular function is sensitive to oxidative stress, and reactive oxygen species (ROS) are highly implicated in the aetiology of male infertility in experimental animals and man (Aitken *et al.,* 2004; Agarwal and Sekhon, 2010; Turner and Lysiak, 2008). Both ascorbic acid (AA) and alpha-tocopherol/vitamin E (VE) are potent hydrophilic and lipophilic antioxidant, respectively (Padayatty *et al.,* 2003). They stabilize cell membranes by preventing lipoperoxidation of cytomembranes by scavenging or quenching of ROS, thereby protecting the cells from oxidative damage (Traber and Atkinson, 2007).

1.2 STATEMENT OF RESEARCH PROBLEM

Dog breeding has assumed an increasingly profitable economic venture in Nigeria by providing means of livelihood to many breeders in cities (Stanley, 2003). Therefore, factors that negatively affect the success of dog breeding, including reproductive problems and diseases such as conception failures, abortions, infertility, foetal resorption, need to be investigated (Carmichael and Shin, 2004).

Spermatogonial stem cells are the foundation of spermatogenesis, which is in turn the backbone for semen production and male fertility (Tegelenbosch and de Rooij, 1993). Peroxidation of sperm membrane lipids is a major cause of loss of motility and fertilizing ability of human and mammalian spermatozoa (Salamon and Mexwell, 1995). Under normal physiological conditions, semen contains antioxidants, including AA, VE, catalase, glutathione peroxidase and superoxide dismutase that prevent lipid peroxidation and consequently excessive peroxide formation (Potts and Pasqualotto, 2003). However, the rate of AA synthesis in the liver tissue is lower in dogs compared to other animal species (Chatterjee *et al.,* 1975), and the endogenous antioxidative capacity of semen may be insufficient under stressful and disease conditions. The susceptibility of spermatozoa to oxidative damage is attributed to high concentration of spermatozoa per unit volume of seminal fluids (Aitken *et al.,* 1989) and the ability of spermatozoa to generate ROS (Zini and Sigman, 2009). Therefore, it is particularly susceptible to peroxidative damage, with subsequent loss of membrane integrity, impaired cell function and decreased motility of spermatozoa (O'Bryan *et al.,* 2000). The uncontrolled production of ROS by defective spermatozoa may have detrimental effect on sperm function (Baumber *et al.,* 2000).

Effects of several antioxidants added to diluted bull semen (Bilodeau *et al.,* 2001), cats (Buff *et al.,* 2001), poultry (Cerolini *et al.,* 2006), humans (Calamera *et al.,* 2001), horses (Ball *et al.,* 2001), rams (Yildiz and Daskina, 2004), boars (Chan *et al.,* 2005), rats (Cocco *et al.,* 2005) and dogs (Ceylin and Serin, 2007; Michael *et al.,* 2009) with various controversial results have been reported. However, to the best of my knowledge, no reports have addressed the effects of AA and VE on the semen quality of the mixed-breed dogs in Nigeria *in vivo*. This study was designed to evaluate the effects of AA, VE and their combination on the reproductive and haematological parameters of sexually matured Nigerian mixed-breed dogs in Zaria *in vivo*.

1.3 JUSTIFICATION OF THE STUDY

Sperm membranes must be protected by highly efficient antioxidant system to prevent peroxidative damage during *in vitro* and *in vivo* storage (Yousef *et al.,* 2002). It has been reported that the beneficial effect of antioxidant vitamins such as AA and VE in improving fertilisation was possibly due to a reduction in lipid peroxidation (Burton and Ingold, 1986; Yousef *et al.,* 2002). Ceylan and Serin (2007) reported an increase in the percentage of live, acrosome-intact spermatozoa during storage of dog semen at 5^0C when AA was added to the extender than when not added. This may imply that AA inhibits peroxidation of membrane lipids during storage and, thus, has protective effects on sperm membranes. It has also been demonstrated that VE is the primary component of the antioxidant system of the spermatozoa (Wang and Quinn, 1999), and is one of the major membrane protectants against ROS and lipid peroxidation (Yousef *et al.,* 2002). Therefore, VE contributes significantly to the higher sperm concentration (Yousef *et al.,* 2002). The effects of AA and VE on the semen quality and serum testosterone level in the mixed-breed dog in Nigeria has

not been reported in the available literature to the best of our knowledge. There is therefore the need to carry out this investigation, as the results of such investigation may shed light on the role of antioxidant vitamin supplementation in improving semen quality and fertility of Nigerian mixed-breed dogs.

1.4 GENERAL AIM OF THE STUDY

The general aim of this study was to determine the effects of AA and/or VE on semen characteristics, serum testosterone levels and some haematological parameters in sexually matured mixed-breed dogs in Zaria.

1.5 SPECIFIC OBJECTIVES OF THE STUDY

The specific objectives of this study were to determine the effects of:

I. ascorbic acid (AA) on semen characteristics, serum testosterone levels and some haematological parameters of mixed-breed dogs in Zaria.

II. vitamin E (VE) on semen characteristics, serum testosterone levels and haematological parameters of mixed-breed dogs in Zaria.

III. combined administration of AA and VE on semen characteristics, serum testosterone levels and haematological parameters of mixed-breed dogs in Zaria.

1.6 RESEARCH HYPOTHESES

Ho_1: The administration of AA has no effect on physical characteristics of semen, serum testosterone levels and haematological parameters in Nigerian mixed-breed dogs in Zaria.

Ho$_2$: The administration VE has no effect on physical characteristics of semen, serum testosterone levels and haematological parameters in Nigerian mixed-breed dogs in Zaria.

Ho$_3$: The combined administration of AA and VE has no effect on physical characteristics of semen, serum testosterone levels and haematological parameters in Nigerian mixed-breed dogs in Zaria.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

2.1.1 Reproductive Pattern in Dog

Domestic dogs attain sexual maturity between the age of six to twelve months for both males and females, although this can be delayed until up to two years old in some large breeds (Feldman and Nelson, 1996). Female dogs will attain first oestrus (puberty), thereafter, they will experience subsequent oestrus biannually, during which they become mentally and physically receptive to copulation and the body prepares for pregnancy (Anonymous, 2002). The ova survive and are capable of being fertilized for a week after ovulation, it is therefore possible for a female to mate with more than one male (Feldman and Nelson, 1996; Dewey and Bhagat, 2002) and be fertilized. Domestic male dogs may breed all-year-round and be ready to mate in the presence of females in estrus (Asa, 1998), however, seasonal variations have been described in some reproductive traits such as semen quality (Sekoni and Gustafason, 1980; Casao *et al.,* 2010), sperm output and concentrations of glyceryl phosphorylcholine in seminal plasma (Verstegen *et al.,* 2002), and serum testosterone (Amann, 1986).

Dogs bear their litters between 56 to 72 days after fertilisation, with an average of 63 days (Concannon, 1983). An average litter consists of about six puppies, but this number may vary widely depending on the breed of the dog. Toy dogs generally produce from one to four puppies in each litter, while much larger breeds may produce as many as twelve (Concannon, 1986).

There is dearth of information on reproductive patterns of dogs under tropical conditions, and the influence of antioxidants on the fertility of dogs, particularly in developing countries where knowledge of reproductive patterns in free-roaming and owned dogs is important in order to prevent reproductive problems in these troops of dogs.

2.1.2 Mating Behaviour of Dog

Canine copulation involves the male mounting the female from behind, as with most tetrapods (Natalie and DE Vito, 1988). When a female dog (bitch) is in proestrus i.e. the preparatory period, she is unreceptive, which she may indicate by sitting, lying down, snapping, or otherwise be uncooperative. The male dog will sniff the female's vulva and continue to examine her rear. When she becomes receptive, known as 'heat', or the estrus period, she will stand still and hold her tail to the side, a stance referred to as "flagging", before allowing the male to mount her from behind whilst attempting penetration with his penis (Beach, 1976; Bekoff and Diamond, 1976).

The canine penis is usually not erect at the time of penetration, it is only able to penetrate the female because it has a narrow bone called the baculum (os penis), a feature of most placental mammals, except in humans, elephants, rabbits, hyena and whales, among others (Beresford and Burkart, 1977; Gilbert, 2001). After penetration, the male will often hold the female tighter and thrust faster, and it is during this time that full erection is achieved (Pal, 2003). Similar mounting behavior, which may include pelvic thrusting is common to canines of both sexes. Mounting, with or without thrusting, should not be confused with "copulatory mounting", in which thrusting continues only until a "tie" is achieved (Beach and LeBoeuf, 1967).

Male canines are the only animals that have a locking bulbus glandis or "bulb", a spherical area of erectile tissue at the base of the penis. During copulation, and only after the male's penis is fully erect inside the female's vagina, the bulbus glandis becomes engorged with blood. The female's vagina subsequently contracts and the penis becomes locked inside the female (Bekoff and Diamond, 1976). This is known as "tying" or "knotting". When the penis is locked into the vagina by the bulbus glandis (i.e. when the stud is "tied"), the male will usually lift a leg and swing it over the female's back while turning around; the two animals face opposite directions and stand with their hind ends touching while the penis is locked inside the vagina as ejaculation occurs (Beach and LeBoeuf, 1967). This "tie" which lasts for an average of 25.65 ± 1.43 minutes (Pal, 2011), helps to decrease leakage of semen from the vagina. After some time, the bulbus glandis disengorges, and the mates separate (Chawla and Reece, 2002). A young female dog can become quite distressed when unable to separate during first copulation, and may try to pull away or run. It is often suggested that handlers calm the mating dogs if they show anxiety during the tying stage (Chawla and Reece, 2002).

In the tropics, more female dogs are reported to be receptive to males during late autumn and winter, and both males and females are highly attracted to each other when the females are on heat (Pal, 2011); however, all males are not equally attracted to a particular female (Pal *et al.,* 1999). Some males are not attracted to some particular females (Cox and LeBoeuf, 1977), while some females do prefer the young adults with greater frequency, and for some, older males may be preferred for physical and /or social quality in an environment (Trivers, 1972; Manning, 1985). No successful copulation is

achieved when more than 2 or 3 males are present with a female at a mating ground (Ghosh *et al.,* 1984), this has also been reported in feral cats.

2.2 ASCORBIC ACID

Ascorbic acid (AA) is a water-soluble sugar with antioxidant properties. The L-enantiomer of AA is commonly known as vitamin C. The name ascorbate is derived from the alpha-privative a- (meaning no) and *scorbiticus* (scurvy), the disease caused by a deficiency of AA. At the time of its discovery in the 1920s, it was named hexuronic acid by some researchers (Svirbelf and Szent-Gyorgyi, 1932: Wilson, 1975). AA is found in plants, animals and single-cell organisms (Derek, 1996). The presence of ascorbate is required for a range of essential metabolic reactions in all animals and plants. It is synthesized internally by almost all mammals, but human being is a notable exception. Thus, is widely known that a deficiency in this vitamin causes scurvy in humans (Sauberlich, 1997). When given orally, AA is well absorbed at lower doses, but absorption decreases at higher doses (Scott *et al.,* 2002).

All living animals either synthesize AA, eat it, or may die from scurvy due to lack of the vitamin. Reptiles produce AA in their kidneys. Recent orders of birds (e.g. ostrich, penguin, albatross, flamingo, eagle, parrot, fowl, gull, pigeon, etc) and most mammals produce AA in their livers, where the enzyme L-gulonolactone oxidase is required to convert glucose to AA (McClausky and Elsewood, 1985; Stone *et al.,* 2004). However, the rate of AA synthesis in the liver tissue is lower in the dog compared to that in other animal species (Chatterjee *et al.,* 1975), particularly during stress or rigorous exercise (National Research Council of National Academics, 2006b). Humans, guinea pigs and some other

primates do not have L-gulonolactone oxidase because of genetic defect, and are, therefore, unable to synthesize AA (Stone *et al.,* 2004).

The absorbance of AA occurs in the intestines using a sodium-ion dependent channel. It is transported through the intestine via both glucose-sensitive and glucose-insensitive mechanisms. The presence of large quantities of sugar either in the intestines or in the blood can slow down absorption. Natural and synthetic L-ascorbic acids are chemically identical and there are no known differences in their biological activities or bioavailability (Naidu, 2003; Stone *et al.,* 2004). The main route of elimination of AA and its metabolites is through urine. It is excreted faster and unchanged when high doses are consumed (Naidu, 2003).

2.2.1 Importance of Ascorbic Acid

Ascorbic acid was finally isolated in 1932 and commercially "synthesized" in 1934 (Allen and Burgess, 1950). However, prior to its identification and isolation, the British Navy sailors were using lime juice to prevent scurvy in 1795 (Wilson, 1975).The uses and recommended daily intake of vitamin C are still under investigation, however, with required daily intake ranging from 45 to 95 mg/day, proponents of megadosage propose from 200 mg to more than 2000 mg/day (Food Standards Agency (UK), 200?). As early as 1984 researchers knew that supplementation of drinking water with vitamin C increased the average life span of mice by as much as 20 percent (Massie *et al.,* 1984)

As a component in hydroxylation (Peterkofsky, 1991), AA is needed for the production of collagen in the connective tissue (Kivirikko and Myllyla, 1985; Prockop and Kivirikko, 1995). Some tissues have a greater percentage of collagen, especially: skin, blood vessels,

tendons, ligaments, teeth, bones and cartilages. Hydroxylation allows the collagen molecule to assume its triple helix structure thus making AA essential to the development and maintenance of scar tissue, blood vessels, bones and cartilages (Peterkofsky, 1991). AA is required for the biosynthesis of dopamine, which in turn participates in the biosynthesis of noradrenaline (Kaufman, 1974; Levine *et al.,* 1992) in the nervous system, adds amide groups to peptide hormones to greatly increase their stability (Eipper *et al.,* 1992; 1993), and also modulates tyrosine metabolism (Englard and Seifter, 1986). AA is also required for the synthesis of carnitine (Padayaty and Levine, 2001), a small molecule that is essential for the transport of fat into cellular organelles called mitochondria, where the fat is converted to energy (Carr and Feri, 1999).

The tissues with greatest percentage of AA (over 100 times the level in blood plasma) are the adrenal glands, pituitary, thymus, corpus luteum and retina. The brain, spleen, lung, testicle, lymph nodes, liver, thyroid, small intestinal mucosa, leucocytes, pancreas, kidneys, and salivary glands usually have 10 to 50 times the concentration present in plasma (Yamada *et al.,* 2004; Emadi-konjin *et al.,* 2005).

AA is involved in the metabolism of cholesterol to bile acids, which may have implications for blood cholesterol levels and the incidence of gallstones (Simon and Hudes, 2000). AA is a natural antihistamine and prevents histamine release and increases the detoxification of histamine. Johnston *et al.* (1992) reported that taking 2 grams of vitamin C daily lowered blood histamine levels up to 38 percent in healthy adults in just one week. AA catalyzes other enzymatic reactions involving amidation reaction, necessary for maximal activity of oxytocin, vasopressin, cholecystokinin and alpha-melanotripin (Levine *et al.,* 1999).

AA enhances the availability and absorption of iron from non-heme iron sources (Hallberg, 1981). The reduction of iron by AA has been suggested to increase dietary absorption of non-heme iron (Bendich and Cohen, 1990). It is widely used as a food additive, and tolerated at high doses without apparent side-effects (McClausky and Elswood, 1985; Balz, 2003). Vitamin C is water soluble, with dietary excesses not absorbed, and excesses in the blood rapidly excreted in the urine.

AA has been reported to have anti-cancer properties (Caraballoso *et al.,* 2003; Bjelakovic *et al.,* 2008) by promoting the production of hydrogen peroxide, which is selectively toxic to cancer cells (Chen *et al.,* 2005; 2007; 2008). AA is also reported to be useful in lowering serum uric acid levels, resulting in a correspondingly lower incidence of gout (Choi *et al.,* 2009), and the oxidized version of AA that can cross the blood-brain barrier may reduce neurological deficits and mortality following a stroke (Huang *et al.,* 2001). There is suggestive evidence AA may be useful in the treatment of pneumonia (Hemila and Louhiala, 2007). AA has been shown to stimulate both the production (Jariwalla and Harakeh, 1996) and function (Levy *et al.,* 1996) of leucocytes (white blood cells), especially neutrophils, lymphocytes, and phagocytes. Specific measures of functions stimulated by vitamin C include cellular motility (Anderson *et al.,* 1980), chemotaxis (Levy *et al.,* 1996; Anderson *et al.,* 1980), and phagocytosis (Levy *et al.,* 1996). AA deficiency is detrimental to immune function, resulting in reduced resistance to some pathogens. The effects are most pronounced in cases of physical strain or insufficient dietary intake (Strohle and Hahn, 2009).

Ascorbic acid is also a highly effective antioxidant, acting to lessen oxidative stress (Padayathy *et al.,* 2003), a substrate for ascorbate peroxidase (Higdon, 2007). Even in

small amounts, AA can protect indispensable molecules in the body such as proteins, lipids (fats), carbohydrates, and nucleic acids (DNA and RNA), from damage by free radicals and reactive oxygen species that can be generated during normal metabolism as well as through exposure to toxins and pollutants (for example, cigarette smoke) (Tak *et al.,* 2000). The plasma AA concentration in oxidative stressed patients (<45 μmol/L) is lower than healthy individuals (61.4 – 80 μmol/L) (Schorah *et al.,* 1996). It has been demonstrated that AA controls mood and brain functions. AA ameliorates heat stress and the adverse effects of environmental conditions (Minka and Ayo, 2007; 2008).

Ascorbic acid may also regenerate other antioxidants such as vitamin E (Chen and Chang, 1978; Wenk *et al.,* 2000; Bruno *et al.,* 2006). In this way, the regenerated vitamin E molecule is again available for antioxidative action or it can be stored (Packer *et al.,* 1979; Eichenberger *et al.,* 2004). One recent study of cigarette smokers found that AA regenerated vitamin E from its oxidized form (Bruno *et al.,* 2006), though results in other species are conflicting (Hesta *et al.,* 2008). Burton *et al.* (1990) concluded that the sparing action of AA on vitamin E is of negligible importance in animals that are not oxidatively stressed.

2.2.2 Deficiency of Ascorbic acid

Ascorbic acid is a co-factor in several collagen synthesis reactions (Kivirikko and Myllyla, 1985; Peterkofsky, 1991). Its deficiency, though rare, has been reported to cause the most severe symptoms of scurvy in humans (WHO, 2001; Higdon, 2007) and somewhat similar symptoms in animals (Evans, 1983; University of Maryland Medical Center, 2007). Dietary intake is reported adequate to prevent deficiency symptoms in nearly all cases,

therefore supplementation is not always necessary (WHO, 2001; Shenkin *et al.,* 2006; Woodside *et al.,* 2006)

2.2.3 Sources of Ascorbic Acid

Ascorbic acid is widely distributed in fruits such as Indian gooseberry, black currant, guava, papaya, strawberry, orange, lemon, mango, pineapple, watermelon and other fresh fruits (Hitti, 2006; US Agricultural Research Service, 2007), green leafy vegetables, tomatoes, broccoli, green and red peppers, cauliflower and cabbage (Nadiu, 2003; US Agricultural Research Service, 2007). It is also in abundance in various foods of animal origin such as the raw liver of calf, beef, pork, chicken and lamb, fried, or roasted, or boiled lamb heart, tongue and brain, calf adrenals (Toutain *et al.,* 1997), oysters and human milk, not raw cow milk (Clark, 2007). AA is a labile molecule and it may be lost from foods during cooking or processing (Allen and Burgess, 1950), even though it has the ability to preserve food by virtue of its reducing property.

Synthetic AA is available in a wide variety of supplements viz., tablets, capsules, chewable tablets, crystalline powder, effervescent tablets and liquid form. Buffered AA and esterified form of AA as ascorbyl palmitate are also available commercially (Naidu, 2003). Both the natural and synthetic AA are chemically identical and there are no known differences in their biological activities or bio-availability (Naidu, 2003; Stone *et al.,* 2004). Most of the plants and animals synthesize AA from D-glucose or D-galactose (Chatterjee *et al.,* 1973; 1975). World production of synthesised AA is currently estimated at approximately 110,000 tonnes annually. Main producers have been BASF/Takeda, DSM, Merck and the China Pharmaceutical Group Ltd. of the People's Republic of China.

China is slowly becoming the major world supplier as its prices undercut those of the US and European manufacturers (Patton, 2005).

2.2.4 Health Risks from Excessive Ascorbic Acid

2.2.4.1 As Pro-oxidant

AA behaves not only as an antioxidant but also as a pro-oxidant (Satoh and Sakagami, 1997; McGregor and Biesalski, 2006) when used at high dose (NRCNA, 2006a) and this can generate superoxide and other ROS (Satoh and Sakagami, 1997). AA may also increase DNA damage in lymphocytes (Podmore *et al.,* 1998), but this effect is reduced by an increased vitamin E intake (NRCNA, 2006a).

2.2.4.2 Gastro-intestinal Tract Disturbances

Relatively large doses of AA may cause indigestion, particularly when taken on an empty stomach. However, taking vitamin C in the form of sodium ascorbate and calcium ascorbate may minimize this effect. High doses (thousands of milligrams) may result in diarrhea in healthy adults, as a result of the osmotic water-retaining effect of the unabsorbed portion in the gastro-intestinal tract (similar to cathartic osmotic laxatives) and skin rashes in infants (WHO, 1973). Other symptoms in adults include nausea, vomiting, diarrhoea, flushing of the face, headache, fatigue and disturbed sleep.

2.2.4.3 Disturbances of Cardiovascular System

As AA enhances iron absorption (Flemming *et al.,* 2002), therefore, iron poisoning may occur in individuals with rare iron overload disorders, such as haemochromatosis. A genetic condition that results in inadequate levels of the enzyme glucose-6-phosphate

dehydrogenase (G6PD) can cause patients to develop haemolytic anemia after ingesting very large dosages of AA (Cook and Reddy, 2001).

2.2.4.4 Urinary System Disturbance

There is a longstanding belief among the mainstream medical community that AA causes kidney stones, which is based on little science (Godwin and Tangum, 1998). Although recent studies have found a relationship (Messy *et al.,* 2005), a clear link between excess AA intake and kidney stone formation has not been generally established (Nadiu, 2003). Some case reports exist for a link between patients with oxalate deposits and a history of high-dose AA usage (Mashour *et al.,* 2000).

2.2.4.5 Reproductive Disturbance

High doses of AA (>1000 mg) have been reported to suppress the production of progesterone necessary for the maintenance of a pregnancy during the first month of pregnancy in rats (Ovcharov and Todorov, 1974). This it achieves by promoting oxidation as it causes the accumulation of peroxide and superoxide radicals in the corpus luteum, causing luteolysis (Sawada and Carlson, 1989; Riley and Behman, 1991).

2.2.5 Mechanism of Action of Ascorbic Acid

Ascorbate acts as an antioxidant by being available for energetically favourable oxidation. Many oxidants, typically reactive oxygen species (ROS), such as hydroxyl radical (formed from hydrogen peroxide), contain an unpaired electron and, thus, are highly reactive and damaging to mammals at the molecular level (Burton and Ingold, 1986; Jones *et al.,* 1995). This is due to their interaction with nucleic acid, proteins, and lipids. ROS oxidize ascorbate first to monodehydroascorbate and then dehydroascorbate. The ROS are reduced

to water, while the oxidized forms of ascorbate are relatively stable and un-reactive, and do not cause any cellular damage (Halliwell *et al.,* 1997). The mechanism of action of AA is by scavenging of superoxide anion by forming semidehydroascorbate radical, which is subsequently reduced by glutathione (Palmieri *et al.,* 2009). Ascorbate acts as a reducing agent to reverse oxidation in aqueous solution (Padayatty and Levine, 2001).

2.3 ALPHA-TOCOPHEROL, OR VITAMIN E

Vitamin E (VE) is a fat-soluble vitamin with antioxidant properties. The term vitamin E describes a family of eight antioxidants: four tocopherols (alpha-, beta-, gamma-, and delta-) and four tocotrienols (alpha-, beta-, gamma-, and delta-) (Traber and Atkinson, 2007). Of these, alpha-tocopherol which has the highest bioavailability has been the most studied (Brigelius-Flohe *et al.,* 1999). Synthetically produced alpha-tocopherol contains equal amounts of its eight possible stereoisomers; serum and tissues maintain only four of these stereoisomers (Verhagen *et al.,* 2006).

It has been reported that alpha-tocopherol is the most important lipid-soluble antioxidant, and that it protects cell membranes from oxidation by reacting with lipid radicals, produced in the lipid peroxidation chain reaction (Herrera *et al.,* 2001). However, the importance of the antioxidant properties of this molecule at the concentrations present in the body is not clear, and the need to include vitamin E in the diet may be related to its ability to act as an antioxidant (Brigelius-Flohe, 2009). However, the roles and importance of all the various forms of vitamin E are presently unclear (Atkinson *et al.,* 2007; Brigelius-Flohe and Davies, 2007).

2.3.1 Importance of Vitamin E

Many reports have been made about vitamin E's potential to promote health, prevent and treat diseases (Traber, 2006). The mechanisms by which vitamin E might provide this protection include its function as an antioxidant and its roles in anti-inflammatory processes (Sen *et al.,* 2006), inhibition of platelet aggregation (Glynn *et al.,* 2007), and immune enhancement (Traber, 2007). A primary barrier to characterizing the roles of vitamin E in health is the lack of validated biomarkers for vitamin E intake and status to help relate intakes to valid predictors of clinical outcomes (Verhagen *et al.,* 2006).

The role of vitamin E in animal reproduction has been recognized since early 19th century, as impairment of mammalian fertility has been attributed to deficiency of vitamin E (Castellini *et al.,* 2007). *In vitro* studies have shown that the nutrient inhibits oxidation of low-density lipoprotein (LDL) cholesterol, thought to be a crucial initiating step for atherosclerosis (Stampfer *et al.,* 1993).

Vitamin E might also help to prevent the formation of blood clots that could lead to a heart attack or venous thromboembolism (Glynn *et al.,* 2007). Some researchers have suggested that understanding the potential utility of vitamin E in preventing chronic heart disease might require longer studies in younger participants taking higher doses of the supplement (Blumberg and Frei, 2007).

Vitamin E might also block the formation of carcinogenic nitrosamines formed in the stomach from nitrites in foods and protect against cancer by enhancing immune function (Weitberg and Corvese, 1997). Human trials and surveys that attempted to associate vitamin E intake with cancer incidence have generally been inconclusive. Some research

links higher intakes of vitamin E with a decreased incidence of breast and prostate cancers (Chan *et al.,* 1998), but the evidence is inconsistent.

Age-related macular degeneration (AMD) and cataracts are among the most common causes of significant vision loss in older people and the etiologies are usually unknown, but the cumulative effects of oxidative stress have been postulated to play a role (Lacey, 2005). Thus, nutrients with antioxidant functions, such as vitamin E, could be used to prevent or treat these conditions (AEDS Research Group, 2001; Jacques *et al.,* 2005; Leske *et al.,* 2005).

Over 2 years, treatment with vitamin E and selegiline (a monoamine oxidase inhibitor), separately or together, in patients with cognitive decline and neurodegenerative diseases, such as Alzheimer's disease, significantly delayed functional deterioration and the need for institutionalisation compared to placebo (Morris *et al.,* 2002). More research though is needed to identify the role of vitamin E in the management of cognitive impairment (Isaac *et al.,* 2008).

2.3.2 Vitamin E Deficiency

Frank vitamin E deficiency is rare and overt deficiency symptoms have not been found in healthy people, who obtain little vitamin E from their diets (Verhagen *et al.,* 2006). Premature babies of very low birth weight (<1.5 kg) might be deficient in vitamin E. Vitamin E supplementation in these infants might reduce the risk of some complications, such as those affecting the retina, but they can also increase the risk of infections (Hathcock, 1997). Since the digestive tract requires fat to absorb vitamin E, individuals with fat-malabsorption disorders are more likely to become deficient than those without such disorders, and may require enormous doses of supplemental vitamin E

(approximately 100 mg/kg or 5-10 g/day) (Traber, 2006). Deficiency symptoms include peripheral neuropathy, ataxia, skeletal myopathy (nutritional muscular myopathy), retinopathy, and impairment of the immune response (U.S. Department of Agriculture, 2004). People with Crohn's disease, cystic fibrosis, or an inability to secrete bile from the liver into the digestive tract, for example, often pass greasy stools or have chronic diarrhoea; as a result, they sometimes require water-soluble forms of vitamin E, such as tocopheryl polyethylene glycol-1000 succinate (Traber, 2006). Clinical effects of vitamin E deficiency have been reported in weaned rabbits (Chan *et al.,* 1980) as well as neurological lesions in rats (Southam *et al.,* 1991). Clinical features are even more pronounced in deficiency of selenium or vitamin C, indicating synergism between vitamin E, selenium and vitamin C (Hill *et al.,* 2001).

It has been reported that vitamin E deficiency secondary to abetalipoproteinemia (a rare inherited disorder resulting in poor absorption of dietary fat) causes poor transmission of nerve impulses, muscle weakness, and retinal degeneration that leads to blindness (Tanyel and Mancano, 1997). Ataxia and vitamin E deficiency is another rare, inherited disorder in which the liver's alpha-tocopherol transfer protein is defective or absent. People with ataxia and vitamin E deficiency have such severe vitamin E deficiency that they develop nerve damage and lose the ability to walk unless they take large doses of supplemental vitamin E (Cavalier *et al.,* 1998).

2.3.3 Sources of Vitamin E

In general, food sources with the highest concentrations of vitamin E are vegetable oils, followed by nuts and seeds, avocado, almond, eggs, milk, including whole grains. Adjusting for typical portion sizes, however, for many people the most important sources

of vitamin E include commercial breakfast cereal, spinach and tomato sauce (USDA National Nutrient Database, 2009).

2.3.4 Health Risks from Excessive Vitamin E

2.3.4.1 Haemorrhage

Research has not shown any adverse effects from consuming vitamin E in food (USDA, 2004). However, high doses of alpha-tocopherol supplements can cause haemorrhage and interrupt blood coagulation in animals (Blumberg and Frei, 2007). *In vitro* data suggest that high doses inhibit platelet aggregation (Sesso *et al.,* 2008). Vitamin E can also antagonize vitamin K-dependent clotting factors. As a result, taking large doses with anticoagulant or antiplatelet medications, such as warfarin (Coumadin®), can increase the risk of bleeding, especially in conjunction with low vitamin K intake. The amounts of supplemental vitamin E needed to produce clinically significant effects are unknown, but probably exceed 400 IU/day (Hathcock *et al.,* 2005).

2.3.4.2 Reaction with other Antioxidants

Some people take vitamin E supplements with other antioxidants, such as vitamin C, selenium, and beta-carotene. This collection of antioxidant ingredients blunted the rise in high-density lipoprotein (HDL) cholesterol levels, especially levels of HDL2, the most cardioprotective HDL component, among people treated with a combination of simvastatin (brand name Zocor®) and niacin (Brown *et al.,* 2001; Cheung *et al.,* 2001).

2.3.4.3   Cancer Development, or Regression

Antioxidant nutrients like vitamin E protect cell constituents from the damaging effects of free radicals that, if unchecked, might contribute to cancer development (USDA, 2004;

Doyle *et al.,* 2006). Human trials and surveys that attempted to associate vitamin E intake with cancer incidence have generally been inconclusive (Doyle *et al.,* 2006). Oncologists generally advise against the use of antioxidant supplements during cancer chemotherapy or radiotherapy because they might reduce the effectiveness of these therapies by inhibiting cellular oxidative damage even in the cancerous cells (Doyle *et al.,* 2006; Lewanda, 2008).

2.3.5 **Mechanism of Action of Alpha-tocopherol**

Alpha-tocopherol has been reported to scavenge and remove the free radicals and their intermediates, and then terminates the oxidation reaction (Brigelius-Flohe and Traber, 1999). The phenolic hydroxyl group of the tocopherol reacts with an organic peroxyl radical to form the corresponding organic hydroperoxide and the tocopheroxyl radical (Traber and Sies, 1996). When a molecule of alpha-tocopherol neutralizes a free radical, it is altered in such a way that its antioxidant capacity is lost (Sauberlich, 1997). The alpha-tocopheroxyl radicals produced may be recycled back to the active, reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Simon *et al.,* 2000).

2.4 TESTOSTERONE

Testosterone is an anabolic steroid belonging to the androgen group of hormone. Its secretion is critical for male secondary sexual differentiation, and the Leydig cells are the principal source of testosterone production in the males. Small quantity is produced by the theca cells of the ovaries and the placenta of the females, and the *zona reticularies* of the adrenal glands in both sexes (Ren-Shan *et al.,* 2007). The number of Leydig cells in turn is regulated by luteinizing hormone (LH) and follicle stimulating hormone (FSH). Testosterone plays an important role in health and well-being through the enhancement of

libido, energy, immune function and protection against osteoporosis. Like most hormones, testosterone is supplied to target tissues via the blood, where much of it is transported bound to specific plasma protein, sex hormone-binding globulin (Brooks, 1975). The male animal is said to produce higher amount of testosterone than an adult female, with the female being more sensitive to the hormone from the behavioural point of view (Dabbs and Dabbs, 2000). Testosterone, like other steroid hormones, is derived from cholesterol, and in conjunction with FSH are required to obtain full reproductive potential (Walker and Cheng, 2005). The Leydig cells have been reported to generate ROS through mitochondrial respiration (Chen *et al.,* 2007).VE as an antioxidant can quench lipid peroxidation and eliminate the ROS to protect the Leydig cells from damage. Mather *et al.* (1983) reported that VE could prolong the survival and function of porcine Leydig cells cultured *in vitro*. The density of Leydig cells have also been largely increased in sheep supplemented with VE (Hailing *et al.,* 2011).

2.4.1 Mechanism of Action

All steroid hormones bind to intracellular receptors, and the hormone-receptor complex then binds to DNA in the nucleus, functioning as a transcription factor to alter the rate of formation of particular mRNAs. This results in a change in the rates of synthesis of the proteins coded for, by the genes being transcribed. The resulting increase or decrease in the concentrations of these proteins in the target cells or their rates of secretion by the cells then account for the cell response of the cells to the hormone (Anonymous, 2009).

Testosterone effect is either anabolic or androgenic. The anabolic effects include growth of muscle mass and strength, increased bone density as well as stimulation of growth in height and bone maturation (Bashin *et al.,* 1996), while androgenic functions encompass

maturation of sex organs, especially the penis and formation of the scrotum in the fetus pre- and post-natally, leading to the development of male sex organs and secondary sex characteristics (Mooradian *et al.,* 1987). Two mechanisms of action have been ascribed to testosterone in humans and other vertebrates, they are by activation of the androgen receptor (directly, or as 5-alpha-dihydrotestosterone (DHT)), and by conversion of testosterone to estradiol (Randall, 1994; Hiipakka and Liao, 1998; McPhaul and Young, 2001). Free testosterone is transported to the cytoplasm of the target cells, where it can be bound to the androgen receptor or it can be reduced to DHT by the cytoplasmic enzyme called 5-alpha-reductase before binding. The testosterone-receptor complex then undergoes a structural change that allows it to move into the cell nucleus and bind directly to specific nucleotide sequences of the chromosome DNA (Hormone Response Elements), where they influence transcriptional activity of certain genes, thereby eliciting androgen effects (Randall, 1994). The effect of testosterone is very important in the brain and bone, where its process of aromatisation to estradiol occurs. In the bones estradiol accelerates maturation of cartilage into bone, leading to closure of the epiphysis and conclusion of growth (Meinhardt and Mullis, 2002).

2.5 SPERMATOGENESIS

Spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis (Phillips *et al.,* 2010) and male fertility (Tegelenbosch and de Rooij, 1993). They are heavily outnumbered by the differentiating spermatogonia, spermatocytes, spermatids and sperms that they produce. SSCs are defined like all other stem cells, by their ability to balance self-renewing divisions and differentiating divisions (Hamra *et al.,* 2004). This balance maintains the stem cell pool and meets the proliferative demand of the testes to produce millions of

sperm each day (Bart *et al.,* 2010). The spermatogenic process during the breeding season in sexually-matured mammals is a cyclic, highly organized and coordinated event, in which spermatogonia differentiate into mature spermatozoa (Russell *et al,* 1990a).

Spermatogenesis is the process by which male primary sperm cells undergo meiosis, and produce a number of cells termed spermatogonia, from which the primary spermatocytes are derived. Each primary spermatocyte divides into two secondary spermatocytes (spermatocytogenesis), and each secondary spermatocyte into two spermatids or young spermatozoa. These develop into mature spermatozoa, also known as sperm cells (spermiogenesis). Thus, the primary spermatocyte gives rise to two cells, the secondary spermatocytes, and the two secondary spermatocytes by their subdivision produce four spermatozoa (Foote *et al.,* 1972). The basic function of spermatogenesis is to turn each one of the diploid spermatogonium (having 46 single chromosomes) into four unique set of haploid sperm cells with 23 single chromosomes (Foote *et al.,* 1972).

Among the spermatogonia (all in all, over 1 billion in both testicles) that form the basal layer of the germinal epithelium, there are certain type A cells that divide mitotically and reproduce themselves by homonymous division, whereby the spermatogonia population is maintained. The beginning of spermatogenesis is introduced through the so-called heteronymous division, in which the daughter cells (second group of type A cells) remain bound together by thin bridges of cytoplasm. Through the preservation of these cytoplasmic connections, spermatogonia are inducted into the spermatogenesis process (Dadoune, 2007).

After a further mitotic division, type B spermatogonia also divide themselves mitotically into primary spermatocytes (I). The freshly created primary spermatocytes now enter into

the first meiosis. They then go immediately into the S phase, double their internal DNA, leave the basal compartment and reach the special milieu of the luminal compartment. Following the S phase, these cells attain the complex stage of the prophase of the meiosis and become noticeably visible with a light microscope. In the prophase in every germ cell a new combination of maternal and paternal genetic material occurs. After the long prophase follow the metaphase, anaphase and telophase that take much less time. One primary spermatocyte after 21 days (Foote *et al.,* 1972) yields two secondary spermatocytes (spermatocytogenesis). The secondary spermatocytes go directly into the second meiosis, out of which the spermatids emerge. Since in the secondary spermatocytes neither DNA reduplication nor a recombination of the genetic material occurs, the second meiosis can take place quickly. It lasts only around 12 hours (Foote *et al.,* 1972) and for that reason secondary spermatocytes are rather seldom seen in a histological section. Through the division of the chromatids of a secondary spermatocyte, two haploid spermatids arise that contain only half the original DNA content. In a process lasting several weeks (so-called spermiogenesis) they are transformed into sperm cells with the active assistance of the Sertoli cells (Heller and Clermont, 1963; Foote *et al.,* 1972).

The process of spermatogenesis is highly sensitive to fluctuations in the environment, particularly hormones and temperature. Testosterone is required in large local concentrations to maintain the process, which is achieved via the binding of testosterone by androgen binding protein present in the seminiferous tubules. Testosterone is produced by interstitial cells, also known as Leydig cells, which reside adjacent to the seminiferous tubules. Seminiferous epithelium is sensitive to elevated temperature, and will be adversely affected by temperatures as high as normal body temperature. Consequently, the testes are

located outside the body in a sack of skin called the scrotum to be maintained below body temperature. This is achieved by regulation of blood flow, and positioning towards and away from the heat of the body by the cremasteric muscle and the dartos smooth muscle in the scrotum. Dietary deficiencies (such as vitamins B, E and A), anabolic steroids, metals (cadmium and lead), x-ray exposure, dioxin, alcohol, and infectious diseases will also adversely affect the rate of spermatogenesis (Johnson *et al.,* 1997; Peters *et al.,* 2000).

The complexity of this process necessitates a tight and well-balanced regulatory mechanism, evidenced by the precise duration of spermatogenesis and the presence of defined germ cell associations. These germ cell associations are referred to as stages of spermatogenesis, and they vary in number and duration in a species-specific manner (Weinbauer *et al.,* 2001). Although strain or breed differences can be found in the literature among members of the same species (Russell *et al.,* 1990a), the total duration of spermatogenesis, which takes approximately 4.5 cycles, lasts for 61.9 ± 0.14 days in mongrel (Soares *et al.,* 2009). The duration of the spermatogenic cycle has been generally considered to be constant for a given species. A recent study utilising xenogenic spermatogonial transplantation has demonstrated that the spermatogenic cycle duration is under the control of the germ cell genotype (França *et al.,* 1998), and it lasts for 13.73 ± 0.03 days in the mongrel (Soares *et al.,* 2009).

Tritiated thymidine, a very specific precursor for DNA, is classically utilised as a germ cell marker in order to determine the duration of spermatogenesis (Neves *et al.,* 2002; França and Godinho, 2003). Also, because each Sertoli cell is able to support only a limited number of germ cells, in a species-specific manner, the number of Sertoli cells, established before puberty in mammals, and Sertoli cell efficiency are the best indicators of

spermatogenic efficiency (daily sperm production per gram of testis) (Johnson, 1995; França and Russell, 1998).

Germ cell loss (apoptosis) plays an important role in the seminiferous epithelium homeostasis by limiting the number of sperms produced. This occurs mainly during meiosis, through the elimination of germ cells that are defective or carry DNA mutations, and during the spermatogonial phase in a process named cell-density regulation (de Rooij and Russell, 2000; Young and Nelson, 2001; Weinbauer *et al.,* 2001). Overall, germ cell apoptosis results in the loss of up to 75% of the potential number of mature spermatozoa that can be produced by one differentiated type A1 spermatogonia (Lee *et al.,* 1997; 1999; de Rooij and Russell, 2000).

The Sertoli cell number established during the prepubertal period in mammals determines the final testicular size and the number of sperm produced in sexually mature animals (Orth *et al.,* 1988). Approximately 20–40 million Sertoli cells were observed for most mammalian species studied up to date (Russell *et al.,* 1990b; França and Russell, 1998). Since the number of Sertoli cells is stable in the adult animal and throughout the different stages of the cycle, these cells are used as a reference point to quantify and functionally evaluate the spermatogenic process (França and Russell, 1998; França and Godinho, 2003).

Very little is known about the mechanisms responsible for the regulation of the Leydig cell size, the number of Leydig cells per testis. A dramatic variation in these parameters and the organisation of these cells in the interstitial compartment is found in the literature for different mammalian (França *et al.,* 2000). However, it is already established that Leydig cell volume and Leydig cell capacity to secrete testosterone is positively correlated with the

quantity of smooth endoplasmic reticulum (Zirkin *et al.,* 1980), and a cross-link between the seminiferous epithelium and the Leydig cells is strongly suggested (Habert *et al.,* 2001; Neves *et al.,* 2002).

2.6 OXIDATIVE STRESS AND SPERMATOZOA

When there are more ROS in the body than available antioxidants, the condition is called oxidative stress (Seis, 1985; McGregor and Biesalski, 2006). ROS are highly reactive oxidizing agents belonging to the class of free radicals (Aitken and Fisher, 1994). A free radical is any atom, or molecule, that possesses one or more unpaired electrons (Warren *et al.,* 1987) and, thus, are highly reactive and damaging to mammals at the molecular level (Burton and Ingold, 1986; Jones *et al.,* 1995).

Oxidative stress has an impact on ageing (Michels *et al.,* 2003), cardiovascular disease, hypertension, (Collins *et al.,* 2002), chronic inflammatory diseases (Tak *et al.,* 2000), diabetes (Tak *et al.,* 2000; Mayne, 2003;) as well as on critically ill patients such as with acquired immunodeficiency syndrome (Goodyear-Bruch and Pierce, 2002; Mayne, 2003), cancer (Cameron and Pauling, 1976), individuals with severe burns (McGregor and Biesalski, 2006) and male infertility (Aitken *et al.,* 1992). Individuals experiencing oxidative stress have ascorbate blood levels lower than 45 µmol/L, compared to healthy individual who range between 61.4-80 µmol/L (Schorah *et al.,* 1996). The increased formation of ROS has been correlated with a reduction in the motility (Agarwal *et al.,* 1994; Armstrong *et al.,* 1999) and loss of fertilizing ability of human and mammalian spermatozoa (Aitken *et al.,* 1989).

The susceptibility of spermatozoa to oxidative damage is attributed to high concentration of spermatozoa per unit volume of seminal fluids and the ability of spermatozoa to generate ROS (Jones *et al.,* 1977; Aitken *et al.,* 1989). The uncontrolled production of ROS by defective spermatozoa may have detrimental effect on sperm function (Baumber *et al.,* 2000,). Therefore, it is particularly susceptible to peroxidative damage, with subsequent loss of membrane integrity, impaired cell function and decreased motility of spermatozoa (Shamsi *et al.,* 2008). Spermatozoa are particularly susceptible to ROS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFAs) (Vernet, 2004), and their cytoplasms contain low concentrations of scavenging enzymes (de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996). In addition, the intracellular antioxidant enzymes cannot protect the spermatozoa plasma membrane surrounding the acrosome and the tail, forcing them to supplement their limited intrinsic antioxidant defences by depending on the protection afforded by the seminal plasma (Zini *et al.,* 1993). Oxidative stress not only attacks the fluidity of the sperm plasma membrane, but also the integrity of sperm nucleus (Aitken, 1999).

A strong evidence suggests that a small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (Aitken, 1999). The spermatozoa, like all body cells living in aerobic conditions, constantly face oxygen paradox (De Jonge, 2002). Oxygen is required to support life, but its metabolites such as the ROS can modify cell functions, endanger cell survival, or both (De Jonge, 2002; Zini and Sigman, 2009). Hence, ROS must be continually inactivated to keep only small amount necessary to maintain normal cell function (Powers, 2004).

2.7 LIPID PEROXIDATION IN SPERMATOZOA

Lipid peroxidation is the oxidative deterioration of polyunsaturated fatty acids (PUFAs); that is, fatty acids that contain more than two carbon-carbon double bonds (Halliwell, 1984; 2007). Oxidative stress-induced damage to sperm may be mediated by lipid peroxidation of the sperm plasma membrane, reduction of sperm motility, and damage to the DNA in the sperm nucleus (Saleh and Agarwal, 2002). Lipid peroxidation occurs in two fundamental stages: initiation and propagation (Aitken *et al.,* 1993). The hydroxyl radical (OH⁻) is a powerful initiator of lipid peroxidation (Aitken and Fisher, 1994). Most membrane PUFAs have unconjugated double bonds that are separated by methylene groups. The presence of a double bond adjacent to a methylene C-H bonds weaker and, therefore, hydrogen is more susceptible to abstraction. Once this abstraction has occurred, the radical produced is stabilized by the arrangement of the double bonds, which form a conjugated diene radical that can then be oxidized. This means that lipids which contain many methylene-interrupted double bonds, are particularly susceptible to peroxidation (Blake *et al.,* 1987).

The conjugated dienes rapidly react with O_2 to form a lipid peroxyl radical (ROO-), which abstracts hydrogen atoms from other lipid molecules to form lipid hydroperoxides (ROOH). Lipid peroxides are stable under physiological conditions until they contact transition metals such as iron or copper salts. These metals or their complexes cause lipid hydroperoxidases to generate alkoxyl and peroxyl radicals, which then continue the chain reaction within the membrane and propagate the damage throughout the cell (Halliwell, 1984). Propagation of lipid peroxidation depends on the antioxidant strategies employed by spermatozoa (Simsek *et al.,* 2005). The results of such exhibit an excellent correlation

with the degree to which function is impaired in terms of motility and the capacity for spermocyte fusion (Sidhu *et al.,* 1998). The consequences of lipid peroxidation include damage to membranes, inhibition of enzyme activities and accumulation of reaction products (Ullrey, 1981).

2.8 ASCORBIC ACID AND REPRODUCTION

Vitamin C is important in many metabolic processes, but is not an essential dietary in dogs, as dogs rely on endogenous synthesis from glucose for their AA requirement (NRCNA, 2006a). However, the rate of AA synthesis in the liver tissue is lower in dogs compared to other animal species (Chatterjee *et al.,* 1975). During stress or intense exercise, the requirement of AA may exceed the synthetic capacity of the liver (NRCNA, 2006b) and additional AA may improve stability of other nutrients and protect against oxidative damage (NRCNA, 2006a). Consequently, AA has been supplemented to domestic animals, including dogs, although these animals are capable of AA synthesis (Wang *et al.,* 2001). However, most studies have failed to demonstrate an effect of antioxidant supplementation on performance (Powers, 2004).

In the seminal plasma, AA concentrations are 10-fold higher than in serum (Dawson *et al.,* 1987; Jacob *et al.,* 1992). However, in semen samples exhibiting ROS activity, ascorbate concentrations in the seminal plasma are significantly reduced (Lewis *et al.,* 1997). Its concentration in seminal plasma is positively related to the percentage of morphologically normal spermatozoa, and it has been suggested that AA is a protective vitamin in the epididymis (Thiele *et al.,* 1995). Furthermore, it has been shown that AA protects human spermatozoa against endogenous oxidative DNA damage (Fraga *et al.,* 1991). AA has been used in the management of male infertility (Mathur *et al.,* 1979) and is essential for the

structural and functional integrity of androgen-dependent reproductive organs (Chinoy, 1978; Chinoy et al., 1983).

2.9 VITAMIN E AND REPRODUCTION

In recent years, vitamin E supplements have been widely used in diets of rats for enhancing production, and reproductive performance have been increased several fold (Chinoy and Sharma, 1998). Antioxidants, including vitamin E, have been shown to improve sperm motility and enhance semen quality and fertility of rats (Yousef et al., 2002). Supplementation with vitamin has also been shown to increase total sperm output and sperm concentration (Sonmez et al., 2007) in boars and poultry. Cerolini et al., (2006) have found improvements in sperm quantity and quality with supplemental vitamins C and E, which was due to the fact that VE is the primary component of the antioxidant system of the spermatozoa as reported by Wang and Quinn (1999), and that it is one of the major membrane protectants against ROS and lipid peroxidation (Yousef et al., 2002). Therefore, VE contributes significantly to the higher sperm concentration (Yousef et al., 2002; Naseem et al., 2007). The association of vitamin E deficiency with impaired male reproduction has been well established for more than three decades, and, traditionally, it is called the 'anti-sterility' vitamin (Uzunhisarcikli et al., 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY AREA

The study was carried out in Zaria, Kaduna State. Zaria is in Northern Nigeria and geographically located between latitude 11^0 4 N and longitude 7^0 42 E (Anonymous, 2005), with a total population of 408,198 as at 2006 census (Anonymous, 2010). It has three distinct climatic seasons, which are: the cold-dry (harmattan) season, the hot-dry season and the hot-humid (wet) season (Dzenda *et al.,* 2011).

3.2 EXPERIMENTAL DOGS

Nine (n = 9) sexually matured and clinically healthy, male dogs, aged 2-3 years and average live weight of 20 kg were obtained within Zaria for the purpose of this study. Simple random sampling was used to assign dogs into two groups, made up of an experimental group of five dogs (n = 5), and a control group, comprising four dogs (n = 4).

3.3 HOUSING AND MANAGEMENT

The dogs were housed in the kennels of the Department of Veterinary Physiology and Pharmacology. The kennels was fumigated and disinfected twice, at two weeks intervals, before housing the dogs. Dogs were acclimatized for a month, during which blood and faecal samples were collected for laboratory analyses for complete blood count, haemoparasite and helminth screening. Vaccination, specifically anti-rabies and distemper-hepatitis-leptospirosis-parvovirus-p complex, and treatment of any other disease conditions were carried out. Dogs were fed individually once a day for accurate control of food intake with kitchen left-over and given free access to water. Semen and Blood samples were

collected every other day and on weekly basis, respectively, for a period of one month before the commencement of the experiment to train the dogs and generate baseline data.

3.4 EXPERIMENTAL DESIGN

The experiments were carried out in phases, between May to September. The order in which the vitamins were administered to each experimental group are shown below in Table 3.1.

Table 3.1: The order of administration of ascorbic acid (AA), vitamin E (VE), and combined ascorbic acid and vitamin E (AA+VE) to each experimental group

Phase (n=5)	Antioxidant	Duration of Experiment
First	AA (5 mg/kg)	4 weeks
Second	VE (5 mg/kg)	4 weeks
Third	AA and VE (5 mg + 5 mg/kg)	4 weeks

The vitamins, 5 mg/kg (Chervyakov *et al.,* 1977) were administered orally every morning between 10:00 - 11:00 hr, and sample collection commenced a week after the commencement of treatment. Vitamins were withdrawn for one month before the beginning of the next phase of the experiment. Dogs in the control group were given placebo (5 ml normal saline orally).

The AA (SPARTAN-C[R]) used in this study is manufactured by Kunimed Pharmaceutichem Ltd, Lagos, Nigeria, and the Alpha-tocopherol (EVIOL[R]) by G. A. Pharmaceuticals, S. A., Greece. Both were obtained from reputable pharmaceutical store.

3.5 SEMEN COLLECTION AND EVALUATION

3.5.1 Semen Collection

Ejaculates were collected in the morning between 08:00-10:00 h by penile massage into pre-warmed, sterile and graduated, transparent collection tube as described by Threlfall (2003). Dog was restrained in a standing position as the prepuce was thoroughly cleaned using cotton ball soaked in diluted chloroxylenol (Dettol[R]). Erection was stimulated by gently massaging the penis at the region of the bulb glandis, while the penis was still within the prepuce. The preputial sheath was then pulled/pushed caudally behind the bulbo glandis to expose the slightly erected penis. A collection cone with an attached graduated collection tube was placed over the penis. Full erection was achieved by gently locking the bulb in a fist and applying pressure with forward and backward movement. In some cases, semen began flowing once the male began to thrust back and forth, but most of the dogs stopped thrusting as they began to ejaculate. Pressure was continuously applied until a crystal clear fluid (the 3rd fraction, or prostatic fluid) began to flow into the collection tube. Only the 1st (pre-sperm) and 2nd (sperm-rich) fractions of the ejaculate were collected. The collection cone was then gently slid off the penis. Semen volume was recorded immediately after collection (Chemineau and Cagnie, 1991). Thereafter, the ejaculates were immediately placed in a water bath at 37^{0}C.

3.5.2 Semen Evaluation

Semen characteristics evaluated weekly were semen volume (recorded in ml), semen concentration (X 10^{6}/ml), percentage sperm mass motility (%), percentage live-dead sperm cells (%) and morphological defects. Mass motility was assessed immediately by examining a drop of raw undiluted semen on a pre-warmed slide under light microscope at the magnification of

x 100 (Rota *et al.,* 1995), and graded in percentage as follows: 0 - 20%, very poor; 20 - 40%, poor; 40 - 50%, fair; 50 - 80%, good; 80 - 90%, very good; and 90 - 100%, excellent; by observing hundred cells showing linear, progressive and wavy motion. Sperm concentration was determined using the improved Neubauer haemocytometer after dilution in 0.05 % formal-saline.

Sperm morphology was determined as described by Zemjanis (1970) and the abnormalities were classified as described by Blom (1972). Percentage dead sperm and morphological sperm abnormalities/defects were determined by Eosin-Nigrosin staining on a glass slide (Johnston, 1991; Oettle, 1993). The staining mixture consisted of 1 % Eosin B and 5 % of Nigrosin in 3 % sodium citrate dehydrate solution. One drop of raw semen was added to 1 drop of the stain, thereafter it was mixed thoroughly and a fresh smear made from it. The slide was then examined under x 100 magnification, and at least 100 cells (both stained and unstained) were counted and the percentage of each estimated. The live-dead staining principle is based upon the observation that Eosin B penetrated and stained the dead sperms (they appeared pink), whereas the viable cells repelled the stain (white in appearance).

3.6 BLOOD COLLECTION AND EVALUATION

3.6.1 Blood Collection

Five millimeter (5 ml) of blood was aseptically collected from each dog weekly after semen collection through the cephalic vein using a 5 ml syringe and 21G x $1^{1}/_{2}$ inch needle. The blood was dispensed into plain and EDTA bottles (3 ml and 2 ml, respectively). The 3 ml in plain tube was centrifuged to extract serum used for testosterone determination, and the 2 ml in the EDTA bottles for complete blood count (CBC). Serum samples were stored frozen at -20°C until analysis.

3.6.2 Blood Evaluation

Complete blood count (CBC) was done within 30 - 40 minutes of blood sample collection. Haematological parameters determined include Packed cell volume (PCV) ($^{0}/_{0}$), haemoglobin concentration (Hb) (g/dL), total leucocyte count (WBC) (x 10^{3}/mL) and absolute leucocyte count (x 10^{3}/mL). Haemoglobin concentration (Hb) was calculated using the formula:

Hb = 1/3 x PCV

The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from the values obtained from RBC, PCV and Hb concentration according to Jain (1986). Quantitative determination of serum testosterone level (ng/ml) was done using a testosterone enzyme immunoassay test kit (AccuBind[R] Testosterone EIA by Monobind Inc., USA) as described by the manufacture. The EIA test kit had a coefficient variation of < 4 % and sensitivity of 0.05 ng/mL.

3.7 STATISTICAL ANALYSIS

Results were expressed as mean plus standard error of mean (mean $\pm$ S.E.M). Data obtained were statistically analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test to determine level of significant differences in all the parameters examined. The Statistical Package for Social Sciences (SPSS) computer program (Version 17.0; SPSS, Chicago, USA) was used. Differences with confidence values of $P < 0.05$ were considered to be statistically significant (Daniel, 1991).

CHAPTER 4

RESULTS

4.1 SEMEN CHARACTERISTICS

4.1.1 Semen Volume

The recorded semen volume in the AA-treated group (2.81 $\pm$ 0.33 ml) was significantly higher ($P < 0.05$) than those in the VE-treated, AA+VE-treated and the control groups (VE = 1.47 $\pm$ 0.34 ml; AA+VE = 1.79 $\pm$ 0.20 ml; control = 1.21 $\pm$ 0.11 ml). There was no significant difference ($P > 0.05$) between the semen volumes recorded in the VE-treated and the control groups, but the semen volumes recorded in the AA+VE-supplemented group (1.79 $\pm$ 0.20 ml) and the control group (1.21 $\pm$ 0.11 ml) were significantly different ($P < 0.05$; Figure 4.1).

Figure 4.1: Effect of ascorbic acid (AA), vitamin E (VE) and ascorbic acid and vitamin E (AA+VE) on semen volume of Nigerian mixed-breed dogs

AA: Ascorbic acid, VE; Vitamin E, AA+VE: Ascorbic acid and vitamin E.

[a b] Mean values with superscripts are significantly different ($P<0.05$)

4.1.2 Sperm Mass Motility

The percentage mass motility recorded in the supplemented groups (AA = 82.5 ± 2.81 %, VE = 86.14 ± 1.90 % and AA+VE = 86.88 ± 5.28 %) were significantly higher ($P < 0.05$) than that obtained in the control group (67.72 ± 2.50 %). There were no significant differences in the percentage mass motility of the spermatozoa among all the experimental groups ($P > 0.05$; Figure 4.2).

Figure 4.2: Effects of ascorbic acid (AA), vitamin E (VE) and ascorbic acid and vitamin E (AA+VE) on the sperm motility of Nigerian mixed-breed dogs

AA: Ascorbic acid, VE: Vitamin E, AA+VE: Ascorbic acid and vitamin E.

[a,b] Mean values with superscripts are significantly different (P<0.05)

4.1.3 Semen Concentration

The semen concentrations in the treated groups were significantly higher (P < 0.05) than the value recorded in the control group. The semen concentration obtained in the AA-treated group (308.8 ± 32.44 x 10^6/mL) was significantly lower (P < 0.05) than those obtained in the VE- and AA+VE-treated groups (Figure 4.3; 388.1 ± 28.42 x 10^6/mL and 435.60 ± 16.71 x 10^6/mL, respectively). The highest semen concentration was recorded in the AA+VE-treated dogs, followed by that of the VE- treated dogs. The semen concentrations obtained in the VE-treated and AA+VE-treated groups were also significantly higher (P < 0.05) than that recorded in the AA-treated group (308.8 ± 32.44 x 10^6/mL) or the control (198.5 ± 9.67 x 10^6/mL).

Figure 4.3: Effects of Ascorbic acid (AA), Vitamin E (VE) and combination of ascorbic acid and vitamin E (AA+VE) on semen concentration of Nigerian mixed-breed dogs

AA: Ascorbic acid, VE: Vitamin E, AA+VE: Ascorbic acid and vitamin E.

[a b] Mean values with superscripts are significantly different (P<0.05)

4.1.4 Sperm Viability

The percentage live sperm cells increased significantly ($P < 0.05$) in the VE- and AA+VE- treated groups (91.00 ± 1.07 % and 91.59 ± 0.75 %, respectively) than in the AA-treated group (86.33 ± 1.86 %), but the value recorded in the VE-treated group was not significantly different from that of the control group ($P > 0.05$; 89.40 ± 0.85 %). The percentage live sperm cells recorded in the AA-treated group (86.33 ± 1.86 %) was also significantly lower ($P < 0.05$) than that of the control group (Figure 4.4; 89.40 ± 0.85 %).
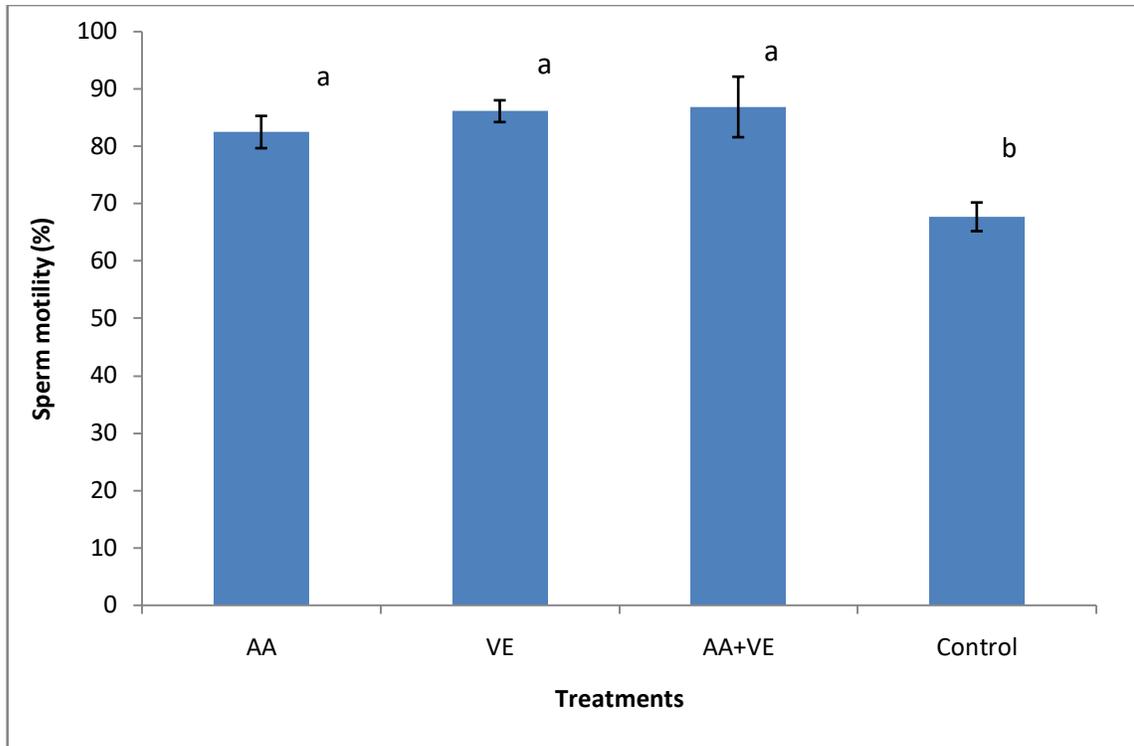
Figure 4.4: Effects of Ascorbic acid (AA), Vitamin E (VE) and ascorbic acid and vitamin E (AA+VE) on percent sperm viability of Nigerian mixed-breed dogs

AA: Ascorbic acid, VE: Vitamin E, AA+VE: Ascorbic acid and vitamin E.

[a b] Mean values with superscripts are significantly different (P<0.05)

4.1.5 Sperm Morphological Defects

The sperm abnormalities observed in this study were mid-piece/cytoplasmic droplets (MPD), bent tail (BT), coiled tail (CT), detached head (DH) and free/detached tail (FT). There were no significant differences ($P > 0.05$) in the overall percentage mean defects observed among the AA- treated group, the AA+VE -treated group and the control (Figure 4.5; 7.02 ± 1.31 %, 6.45 $\pm$ 1.13 % and 6.32 ± 0.79 %, respectively). The VE-treated group recorded the lowest percentage mean of sperm defects ($P < 0.05$; 2.83 ± 0.46 %) among all the groups.
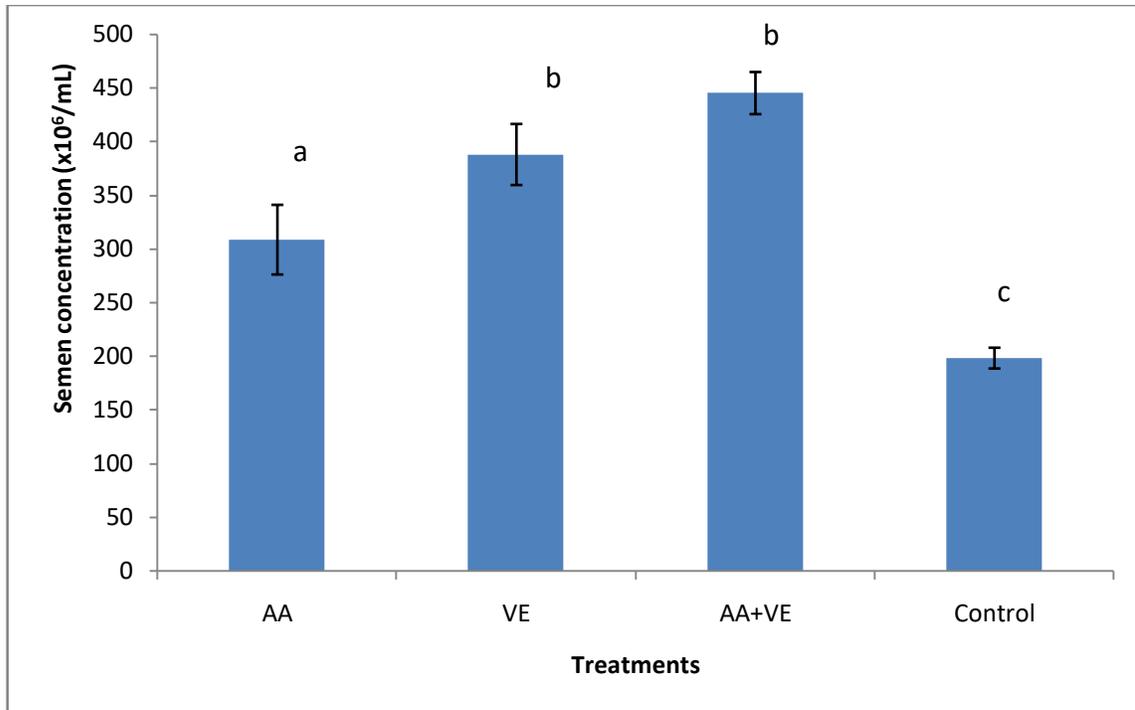
Figure 4.5: Effects of ascorbic acid (AA), vitamin E (VE) and ascorbic acid and vitamin E (AA+VE) on percentage sperm defects in semen of Nigerian mixed-breed dogs

AA: Ascorbic acid, VE: Vitamin E, AA+VE: Ascorbic acid and vitamin E.

[a] [b] Mean values with superscripts are significantly different (P<0.05)

4.2 EFFECTS OF ANTIOXIDANTS ON SERUM TESTOSTERONE LEVEL.

Figure 4.6 shows that the differences in the serum testosterone levels in all the treated dogs and the untreated dogs at the earlier stage of the study were insignificant ($P > 0.05$). The serum testosterone level in the control group was lower ($P < 0.05$) than that obtained in any of the vitamin-supplemented groups. As the week of treatment increased, the testosterone levels significantly rose ($P < 0.05$) in dogs supplemented with either AA (4.51 ± 1.50 ng/mL) or VE (3.14 ± 1.20 ng/mL). There was no significant difference ($P > 0.05$) between the serum testosterone level recorded in the AA+VE-treated group (1.18 ± 0.20 ng/mL) and that of the control group (1.18 ± 0.34 ng/mL).
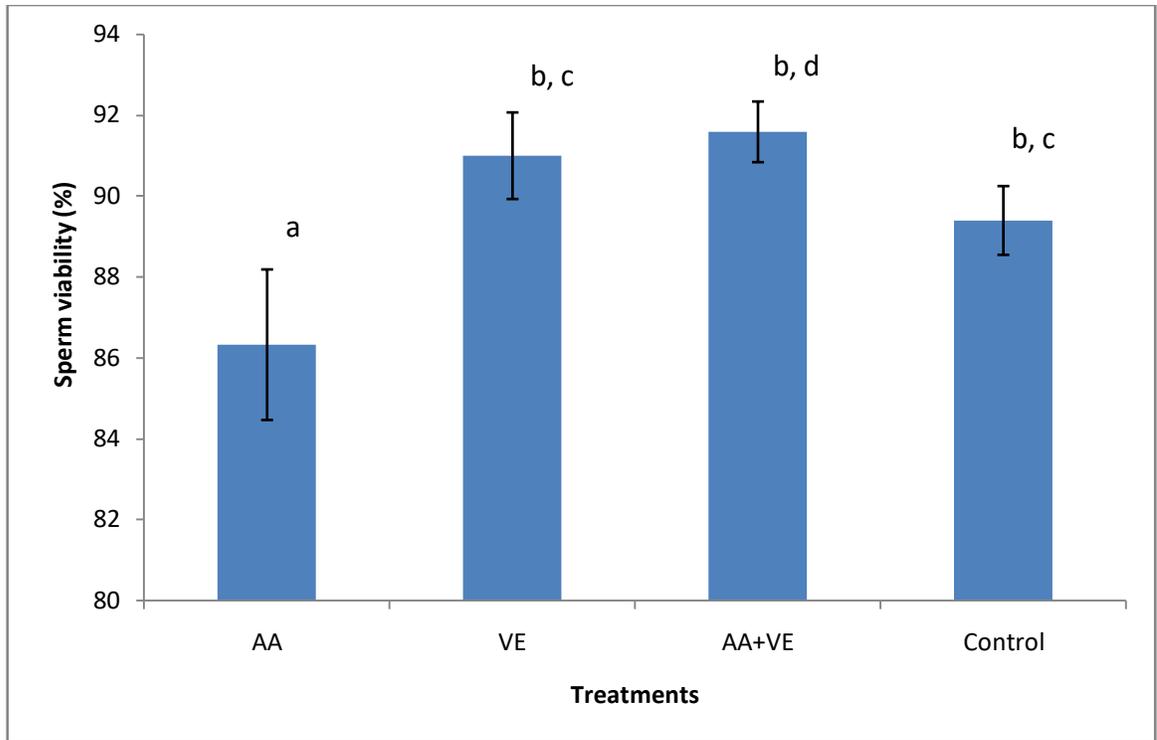
Figure 4.6: Effects of ascorbic acid (AA), vitamin E (VE), and ascorbic acid and vitamin E (AA+VE) on serum testosterone concentration in Nigerian mixed-breed dog

4.3 EFFECTS OF ANTIOXIDANTS ON HAEMATOLOGICAL PARAMETERS

There were significant differences ($P < 0.05$) in the values of erythrocytic parameters (packed cell volume, haemoglobin concentration and mean corpuscular haemoglobin concentration) recorded in the AA+VE-supplemented group and those in the AA-, VE-treated and control groups. The highest values were recorded in the AA+VE-supplemented group (PCV = 43.67 + 2.62 %; Hb = 14.54 ± 0.54 g/dL; MCHC = 33. 28 ± 0.02 g/dL). The values of PCV, Hb and MCHC obtained in the AA-, VE-treated and control groups were not significantly different ($P >$ 0.05; Table 4.1; PCV: AA = 39.15 ± 1.45, VE = 37.92 ± 0.82 % and control = 37.08 ± 2.64 %; Hb value: AA = 13.02 ± 0.38 g/dL, VE = 12.61 ± 0.27 g/dL and contol = 12.36 ± 0.54 g/dL; MCHC: AA = 33.24 ± 0.02 %, VE = 33.26 ± 0.02 %, control = 33.28 ± 0.01). There were no significant differences ($P>0.05$) in the values of total WBC recorded between the supplemented and unsupplemented groups (AA = 8.56 ± 1.03 x10^3 mL, VE = 7.98 ± 0.88 x10^3 mL; AA+VE = 8.14 ± 0.65 x10^3 mL; control = 8.25 ± 0.23 x10^3 mL), as well as in the absolute leucocyte count values as shown in Table 4.1. No significant differences ($P > 0.05$) were observed also among the three supplemented groups.

Table 4.1: Comparative effect of ascorbic acid, vitamin E, and ascorbic acid combined with vitamin E on haematological parameters of Nigerian mixed-breed dogs (Mean ± SEM)

Parameters	AA (n = 5)	VE (n = 5)	AA+VE (n=5)	CONTROL (n = 4)
PCV (%)	39.15 ± 1.45^{a}	37.92 ± 0.82^{a}	43.67 ± 2.62^{b}	$37.08 \pm \pm 2.64^{a}$
Hb Conc. (g/dL)	13.02 ± 0.38^{a}	12.61 ± 0.27^{a}	14.54 ± 0.54^{b}	12.36 ± 0.88^{a}
MCHC (%)	33.24 ± 0.02	33.26 ± 0.02	33.28 ± 0.02	33.28 ± 0.01
Total WBC $(x10^{6} /mL)$	8.56 ± 1.03	7.98 ± 0.88	8.14 ± 0.65	8.25 ± 0.23
Neutrophils $(x10^{3} /mL)$	5967.8 ± 132.6	5441.3 ± 124.7	5642.7 ± 127.6	5679.40 ± 149.33
Lymphocytes $(x10^{3} /mL)$	1810.2 ± 37.5	1833 ± 30.1	1780 ± 32.4	1827.63 ± 21.58
Monocytes $(x10^{3} /mL)$	398 ± 18.8	433 ± 15.9	434.9 ± 11.1	431.57 ± 32.11
Eosinophils $(x10^{3} /mL)$	284.4 ± 14.8	272 ± 13.9	203 ± 13.0	$259.67 \pm 27\ 56$

AA: Ascorbic acid, VE: Vitamin E, AA+VE: Combined ascorbic acid and vitamin E, PCV: Packed cell volume, MCHC: Mean corpuscular haemoglobin concentration, WBC: White blood cells.

[a,b] Mean values with superscripts within rows are significantly different (P < 0.05)

CHAPTER 5

DISCUSSION

5.1 EFFECTS OF ANTIOXIDANTS ON SEMEN CHARACTERISTICS

The higher semen volume obtained in this study in the AA-treated dogs than in the VE-treated dogs agrees with Audet *et al*. (2004), who reported higher semen volume in AA-supplemented than in VE-supplemented boars. Similarly, increase in semen volume due to AA supplementation have been reported in turkeys (Dobrescu, 1987), broiler breeders (Monsi and Onitchi, 1991) and post-molt cockerels (Khan *et al.,* 2012) when they were supplemented with AA compared to the controls. This increase in semen volume due to AA supplementation may be attributed to the potent hydrophilic nature of AA (Odeniyi and Jaiyeoba, 2007) which favours the production of seminal fluid by pulling water molecules into the accessory glands due to high concentration of AA in the seminal fluid; ten folds the amount contained in the plasma (Nikki *et al.,* 1995; Padayatty *et al.,* 2003).

The significantly higher percentage motility observed in all the treated groups than in the control in this study is in agreement with earlier report by Audet *et al*. (2004) who recorded significantly higher percentage of motile sperm cells in boars supplemented with AA. This may be attributed to the ability of AA to pull water molecules into the accessory glands thereby creating more space for each sperm cell /unit volume of seminal fluid, the effect of which reduced generation of reactive oxygen species by the sperm cells. However, contrary to the observation of the present study, reports from *in vitro* studies have shown that AA does not have any beneficial effect on sperm motility, but reduced the motility of spermatozoa in rams (Bathgate *et al.,* 2008), equine (Dukelow and Graham, 1962; Aurich *et al.,* 1997) and dog semen (Ceylan and Serin, 2007; Michael *et al.,* 2008; 2009). The high

semen motility recorded in the VE-treated dogs than the control observed in this study is in agreement with the findings of Suleiman *et al*. (1996), Yousef *et al*. (2002) and Castellini *et al*. (2007) in men, rats and rabbits, respectively. This may be attributed to the fact that VE reduced reactive oxygen species (ROS) generation in the spermatozoa (Surai, 2000; Surai *et al.,* 2001) thereby inhibiting DNA damage (Lopez-Fernandez *et al.,* 2008; Zini and Sigman, 2009), cytoskeleton alterations (Hinshaw *et al.,* 1986) and prevention of its effects on the sperm axoneme responsible for loss of motility (de Lamirande and Gagnon, 1992) as well as promoting sperm-oocyte fusion (Agarwal and Sekhon, 2010). Conflicting results have been reported when VE was added as an extender in *in vitro* studies on semen motility in different species (Yoshida, 2000). The high percentage motility recorded in the AA$\pm$VE-treated dogs agrees with the finding of Hsu *et al*. (1998) in rats, and this was attributed to the synergism of vitamins C and E (Whitehead and Keller, 2003). Although VE inhibits ROS generation, the availability of AA regenerates the produced VE radical (alpha-tocopheroxyl radical) back to alpha-tocopherol molecule (Wenk *et al.,* 2000) for continuous antioxidative action or it can be stored (Eicheinberger *et al.,* 2004).

The high semen concentration obtained in the AA-treated dogs than the control dogs is in agreement with earlier reports by Donnelly *et al*. (1999), Shittu (2010) and Edeh (2011), who observed an increase in sperm concentration in humans, Wistar rats and Spraque Dawley rats, respectively. This was attributed to the ability of the antioxidant property of the vitamin to protect the testicular tissue from naturally-occurring peroxidation thereby improving the biological function of the Sertoli cells. AA also provides favourable environment for spermatozoa synthesis in the seminiferous tubule and their maturation within the epididymis.

Increased total concentration, due to VE supplementation than in the controls as observed in this study have been reported in boars (Sonmez *et al.*2007), rabbits (Yousef *et al.,* 2003), rams (Luo *et al.,* 2004; Yue *et al.,* 2010) and chickens (Cerolini *et al.,* 2006), and this may be attributed solely to the ability of VE to reduce the amount of total reactive oxygen species (tROS) naturally generated by the spermatozoa (Surai, 2000; Surai *et al.,* 2001) due to high concentration of poly-unsaturated fatty acid (PUFA) within their plasma membranes (Mishra and Acharya, 2004; Krishnamoorty *et al.,* 2007). The generated ROS have been reported to impair testicular functions, which has negative effects on semen characteristics (O'Bryan *et al.,* 2000). Dietary supplementation with VE has also been reported to increase the density of Sertoli cells responsible for spermatogenesis in sheep than in the control (Hailing *et al.,* 2011).

The highest value of semen concentration obtained in the AA+VE-treated dogs concords with the findings of Hsu *et al.* (1998) and Shittu (2010) in rats, and may be attributed to the high plasma concentration of AA, due to both endogenous synthesis and exogenous supplementation which promoted the synergistic effect of its combined use with VE. VE has been reported to be regenerated by AA after its reduction (Wenk *et al.,* 2000).

This study has demonstrated that oral VE and VE+AA supplementations in dogs increased the percentage cell viability of sperms than in the AA-treated dogs. The prevention of oxidative reduction by the sperm cells due to high VE in levels of the major PUFAs in humans (Kessopoulou *et al.,* 1995), in boar (Cerolini *et al.,* 2000) and dog (Hatamoto *et al.,* 2006) has been reported as being responsible for increase in spermatozoa viability. However, reported *in vitro* studies in porcine (Dukelow and Graham, 1962), bovine

(Beconi *et al.,* 1993; Klinc, 2005), boar (Grossfeld *et al.,* 2008) and dog (Michael *et al.,* 2009) semen did not concord with the present study.

The percentage live spermatozoa in the AA-treated dogs was significantly low as compared to the controls and other treated groups. This may be because AA begin to act as pro-oxidant and increase lipid peroxidation in high plasma concentration as a result of the endogenous synthesis and exogenous supplementation (Chen *et al.,* 2007). Even though, low doses were used in this study (5 mg/kg/day) compared to previous reports where higher doses have been used (Donoghue *et al.,* 1993; Piercy *et al.* 2000; Marshall *et al.* 2002) (daily doses between 500 mg- 1000 mg per dog), the National Research Council of National Academics (2006a) claimed that the pro-oxidant effect can be reduced by an increased VE intake, which thus explain why the percentage live spermatozoa value was highest in the AA+VE-treated dogs in this study. The observed high percentage viability in the semen of the AA+VE-treated dogs also demonstrated that AA can act synergistically with other protective compounds, including alpha-tocopherol as reported by Whitehead and Keller, 2003.

The least number of sperm morphological defects recorded in the VE-treated group in this study agrees with earlier reports in humans (Kessopoulou *et al.,* 1995) and dogs (Hatamoto *et al.,* 2006) subjected to stress. This may be attributed to the ability of VE to protect and improve the sperm cell plasma membrane integrity (de Lamirande and Gagnon, 1992; Saravia *et al.,* 2005). Similar finding was observed by Michael *et al.* (2009) who reported in an *in vitro* study that all antioxidants tested (taurine, catalase, vitamin C, vitamin E, N-acetyl-L-cysteine and vitamin B12) showed higher percentages of spermatozoa with normal morphology than the control, except vitamin C. This was attributed to significant

reduction in the values of total ROS (tROS) recorded when these antioxidants were used as part of semen extender. The highest value of deformity recorded in the AA-treated dogs may be attributed to the fact that AA supplementation increases the plasma concentration of AA (Scott *et al.,* 2002), and higher dosage in addition with the endogenous synthesis may lead to pro-oxidant effect of AA (Chen *et al.,* 2005). It is worth noting that the dogs in this study were not subjected to any form of stress besides that of handling during sample collection.

5.2 EFFECTS OF ANTIOXIDANT ON TESTOSTERONE LEVEL

Significant increase in the level of testosterone observed in the AA-treated dogs than in VE-, AA+VE-treated and control groups in this study agrees with report of Shittu (2010), who reported increase in testosterone concentration when Wistar rats were treated with AA than with vitamin E, or when the two vitamins were combined. This may be attributed to the ability of AA to lower cortisol levels (Whitehead and Keller, 2003), thus allowing the body to produce more testosterone, and like zinc, AA reduces the aromatase enzyme that converts testosterone into oestrogen. Increase in serum testosterone levels due to VE supplementation in the present study has been similarly reported in rats (Umeda *et al.,* 1982) and rams (Rekkas *et al.,* 2000). This was attributed to ability of VE to apparently increase the potency of LH thereby increasing its effect on the Leydig cells (Umeda *et al.,* 1982) and, thus, increasing testosterone production.

5.3 EFFECTS OF ANTIOXIDANTS ON HAEMATOLOGICAL PARAMETERS

No significant differences were observed in the AA-, VE-treated and untreated groups in this study. The findings in this study agrees with that of Hesta *et al*. (2009), who also

reported no clear effect of AA and VE supplementation at dietary level on haematological parameters of healthy adult dogs at rest. Olajide (2011) also reported that AA exerted no significant effects on the haematology of goats. Similar observations were made in stressed, transported cattle (Knowles *et al.,* 1999), racing pigeons (Scope *et al.,* 2002) and in oxidatively stressed guinea pigs (Obianime and Aprioku, 2011). However, the relatively higher but not significant increase in values of RBC and other erythrocytic parameters (haemoglobin concentration and mean corpuscular haemoglobin concentration) obtained in the three supplemented groups in this study may be attributed to the ability of AA to enhance the availability and absorption of iron from non-haeme sources (Hallberg, 1981), and in its reduced form to improve absorption of iron from the gut (Iqbal, 2004), thereby increasing the serum concentration of iron essential for haeme synthesis (Cook and Reddy, 2001). AA has also been reported to maintain the integrity of erythrocyte membranes (Candan *et al.,* 2002). VE has been reported to enhance the stability of membranes of blood cells - the erythrocytes, leucocytes and platelets, thus providing protection from free-radical attacks (Pinelli-Saavedra, 2003). Adenkola (2010) also recorded no significant difference in the values of haematological parameters in AA-supplemented and non-supplemented pigs before embarking on a journey.

The results of the present study demonstrated the synergistic effectiveness of vitamins C and E when administered together, which may explain the higher percentage of PCV obtained in the AA+VE-supplemented dogs than those supplemented with only AA or VE. This may be due to continuous regeneration of VE by AA (Wenk *et al.,* 2000; Bruno *et al.,* 2006). AA is also present at moderately high plasma concentration as a result of its

endogenous synthesis (Chatterjee *et al.,* 1975; NRCNA, 2006a) in addition to its exogenous supplementation.

The present study showed no significant changes in the total WBC count and absolute leucocyte counts of the treated and control groups. The result of this study agrees with that of Hesta *et al.* (2009) and Olajide (2011) who reported no evidence of a clear change in the leucocyte values by AA and/ or VE supplementation in healthy dogs and goats, respectively. All values recorded in the present study were within normal range of haematological parameters for the normal dog at rest.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

From the findings of this study, some indications for promoting the Nigerian mixed-breed male dog fertility can be summarized as follows:

1. Vitamin C, due to its hydrophilic nature, increased only the semen volume.

2. Semen quality (motility, concentration, viability and morphology) may be improved for optimum fertility by supplementing with vitamin E, or its combination with vitamin C, rather than vitamin C alone.

3. Vitamin C alone was able to improve the production of testosterone than when combined with vitamin E, or vitamin E alone.

4. The combined antioxidant vitamins (C + E) increased the PCV and Hb concentration.

5. The two antioxidant vitamins (C and E) administered either alone or in a combination did not induce any clear change in leucocyte counts of healthy adult mixed-breed dogs at rest.

6.2 CONCLUSION

The present study has shown that supplementation with antioxidant vitamin E, or its combination with vitamin C in stud dogs intended for breeding in the Northern Sahel Savannah zone of Nigeria may promote spermatogenesis and reduce spermatozoa damage/death, caused by oxidative stress, thereby promoting morphologically normal sperm cells.

6.3 RECOMMENDATIONS

1. The Nigerian Mixed-breed dogs should be supplemented with vitamin C (cheap and obtainable across the counter) in combination with vitamin E (100 mg daily) to improve reproductive performance.

2. Further studies should be carried out using different dosage regimen of the antioxidants   for a longer period than that of the present study.

3. Further studies should also be conducted to determine the fertilizing ability of the semen of the Nigerian mixed-breed dog.

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APPENDIX I: Comparative effects of ascorbic acid, vitamin E, and ascorbic acid combined with vitamin E on semen characteristics of Nigerian mixed-breed dogs (Mean ± SEM)

Semen characteristics	AA (n = 5)	VE (n = 5)	AA+VE (n = 5)	CONTROL (n =4)
Semen volume (mL)	2.81 ± 0.33^a	1.47 ± 0.34^b	1.79 ± 0.20^c	1.21 ± 0.11^b
Sperm motility (%)	82.5 ± 2.81^a	86.14 ± 1.90^a	86.88 ± 5.28^a	67.72 ± 2.5^b
Semen concentration (x 10^6/mL)	308.8 ± 32.44^a	388.1 ± 28.42^b	445.3 ± 19.63^b	198.5 ± 9.67^c
Sperm viability (%)	86.33 ± 1.86^a	91.00 ± 1.07^b	91.59 ± 0.75^b	$89.40 \pm 0.85^{a,b}$
Sperm defects (%)	7.02 ± 1.31^a	2.83 ± 0.46^b	6.45 ± 1.13^a	6.32 ± 0.79^a

AA: Ascorbic acid, VE: vitamin E, AA+VE: Ascorbic acid combined with vitamin E

Superscripts a, b = Mean values with different superscripts within rows are significantly different ($P<0.05$)

Testosterone Enzyme Immuno-Assay

The Testosterone EIA is based on the principle of competitive binding between Testosterone in the test specimen and Testosterone-HRP conjugate for a constant amount of rabbit anti-Testosterone. In the incubation, goat anti-rabbit IgG-coated wells are incubated with 10 µI of Testosterone standards, controls, patient samples, 100 µI Testosterone-HRP conjugate reagent and 50 µl rabbit anti-Testosterone reagent at 37^0C for 90 minutes. During the incubation, a fixed amount of HRP-labeled Testosterone competes with the endogenous Testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific Testosterone antibody. Thus, the amount of Testosterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of Testosterone in the specimen increases.

Unbound Testosterone peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB Reagent is then added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled Testosterone in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The Testosterone concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve.

Assay Procedure

1)   Secure the desired number of coated wells in the holder

2)   Dispense 10 µl of standards, specimens and controls into appropriate wells.

3)   Dispense 100 µl of Testosterone-HRP Conjugate Reagent into each well.

4)   Dispense 50 µl of rabbit anti-Testosterone reagent to each well.

5)   Thoroughly mix for 30 seconds. It is very important to mix them completely.

6)   Incubate at 37^0C for 90 minutes.

7)   Rinse and flick the micro-wells 5 times with distilled or deionized water. (Please do not use tap water).

8)   Dispense 100 µl of TMB Reagent into each well. Gently mix for 10 seconds.

9)   Incubate at room temperature (18-25^{0}C) for 20 minutes.

10)   Stop the reaction by adding 100µl of Stop Solution to each well.

11)   Gently mix 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.

12)   Read absorbance at 450 nm with a microtiter well reader <u>within 15 minutes</u>.

Calculation of Results

1)   Calculate the mean absorbance value (A_{450}) for each set of reference standards, controls and samples.

2)   Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on a linear-linear graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.

3)   Use the mean absorbance values for each specimen to determine the corresponding concentration of Testosterone in ng/ml from the standard curve.

4)   Any values obtained for diluted samples must be further converted by applying the appropriate dilution factor in the calculations.

Determination of Total and Differential Leukocyte Count

White blood cell (WBC) count was done using the Haemocytometer method as described by Schalm *et al.,* 1975.

Procedures

1. Total Leucocyte Count

A white blood cell diluting pipette fitted with rubber and plastic mouth piece was filled with the blood sample to the 0.5 mark. The excess blood clinging to the exterior of the pipette was wiped off using a piece of tissue paper. The pipette was then dipped in a bottle containing the WBC diluting fluid (2% glacial acetic acid coloured with 2 drops of gentian violet), this was drawn to the 11 mark. The blood and the diluting fluid were then adequately mixed by horizontal agitation; the red blood cells in the sample were lysed by the acetic acid. Six drops of the diluting fluid was removed by releasing the thumb intermittently. The Haemocytometer was then charged by capillary action. This was then examined under x 40 objective of the microscope. The leukocytes were counted in the 4 large squares. To obtain the total leukocyte count, the value got was multiplied by the factor of 50.

2. Differential Leucocyte Count

A drop of blood was put on a clean glass slide and another clean glass slide was used as a spreader by a slow contact with the drop of blood at 45^{0} to enable capillary spread of blood

along the edge of the slide, and in an aeroplane fashion, changing degree to about 300 progressively making a smear with 3 distinct layers, head, body and tail. The smear was allowed to dry, labeled and then fixed in methanol for 5 minutes. It was removed, allowed to dry and stained with Wright Giemsa (Bayer Corp., Diagnostic Division, Elkhart, In, USA) for 30 minutes. Differential leucocyte counts were carried out under the microscope oil-immersion objective and 100 cells were counted per slide using straight-edge method as described by Schalm *et al.* (1975) to give the relative leucocyte counts. The absolute leucocyte count was calculated using the percentage relative value and the total leucocyte counts.