SPERMATOZOAL QUALITY OF CRYOPRESERVED CANINE SEMEN USING DIFFERENT EXTENDERS AND CHLOROQUINE PHOSPHATE

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A THESIS SUBMITTED TO THE DEPARTMENT OF ANIMAL SCIENCE FACULTY OF AGRICULTURE AND FORESTRY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE UNIVERSITY OF IBADAN.

FEBRUARY, 2012.

CERTIFICATION

I certify that this work was carried out by Mr. Jovi Richard OTITE of the Department of Animal Science, University of Ibadan, Ibadan, Nigeria under my supervision.

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DEDICATION

This work is dedicated to my parents Prof. and Dr. Mrs. Onigu Otite, my wife Yohi and son Aman.

ACKNOWLEDGMENT

My sincere gratitude goes to my supervisor, Prof. D.O. Adejumo for his good advice, guidance and supervision of this project.

I owe a lot of appreciation to my wife Yohi Mersha Otite, my parents Prof. and Dr. Mrs Otite as well as other members of my family for all their support throughout the span of this work.

I wish to express my gratitude to Prof. G.N. Egbunike, Prof. A.O. Akinsoyinu, Prof. A.D. Ologhobo, Dr. O.A. Sokunbi, Dr. A.O. Ladokun, Dr. Dele Ojo, Miss Temitope Lawal, Dr. O.A. Ogunwole, Dr. O.J. Babayemi, Mr. O. Alaba, Dr. A.B. Omojola, Dr. O.A. Adebiyi, Mr. O.O. Aiyepeku and Mr. O.H. Osaiywvu for their assistance in making this work a success.

I also wish to express my sincere gratitude to Prof. O.O. Tewe, Dr. A.E. Salako, Dr. O.A. Abu, Mr. Akin Adamu, Dr. Kilani, Mr. Zhattu Bani and Mr. Daniel Udofia.

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ABSTRACT

Sub-zero degree preservation is critical for effective semen quality extension in artificial insemination of animals. The use of certain drugs and extenders have been reported to enhance the quality of semen in cattle and swine. This is yet to be reported in canines. The effects of different extenders and chloroquine phosphate on the quality of cryopreserved semen of two exotic dog breeds were therefore investigated.

Six studs made up of three Rottweilers and three German Shepherd Dogs (GSD) aged 2 to 3 years, weighing 38 to 40kg, were purposively selected through a spermatogram pilot study. They were then assigned to three replicates of two dogs each, in a completely randomised design. Ejaculates from each stud were analysed for volume (V), Concentration (C), Mass Activity (MA), Progressive Motility (PM), Live to Dead Ratio (LDR), Percentage Abnormality (PA), DNA Integrity (DI), Acrosome Integrity (AI) and Survival Time (ST). Individual ejaculates were diluted (100 million spermatozoa/ml) in Sodium Citrate Glycine Extender (SCGE); Coconut Water Extender (CWE); Skim Milk Extender (SME) and *Hibiscus sabdarifa* (*Zobo* Extender (ZE)). These samples were preserved at 28^{o}C and 4^{o}C and analysed hourly until zero percent motility was observed. The extended semen samples were cryopreserved using liquid nitrogen. The extended semen samples were then subjected to post preservation evaluation. Chloroquine Phosphate (CP), 0.2ml of 3mM, was added to portions of the cryopreserved samples and also evaluated. Data were analysed using descriptive statistics and ANOVA at $p < 0.05$.

Effects of breeds on all the dependent variables were not significant, hence the results were pooled. The SME had optimum significant values of 71.2% PM, 4.0 hrs ST, 3.7% AI, 9.9% PA and MA of 77.5% and 76.3% at 28^{o}C and 4^{o}C, respectively. The highest initial values of MA were observed in ZE (60.0%) which had the greatest PM of 90.3% and 91.9% at 28^{o}C and 4^{o}C respectively. The SCGE had the least values of 30.9% PM, 26.3% MA and 0.9 hrs ST, and also recorded significantly higher values of 15.3% PA and 8.5% AI. Although SCGE had the highest DI (2.0%), the apparent differences in values were not however significant compared with those obtained for CWE, SME and ZE. The AI had a 77.0% higher chance of being affected than DI (23%). The inclusion of CP had significant effects on the initial MA in SCGE (10.8%), CWE (58.3%) and SME (66.7%) but not in ZE (64.2%). Similarly, CP significantly influenced the initial PM in SCGE (12.5%) and CWE (68.0%). The effects of CP on PA were significant only in SME (10.9%) and ZE (13.0%) but similar in SCGE (18.5%) and CWE (15.9%). The CP however significantly affected the survival time of SCGE (0.3hrs), CWE (1.9hrs), SME (2.7hrs) and ZE (2.2hrs).

Skim milk extender was the best extender for cryopreservation in dogs. Addition of Chloroquine phosphate just before insemination enhanced the fertilising potential of cryopreserved canine semen.

Keywords: Semen extenders, Cryopreservation, Canine semen evaluation.

Word count: 468

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Dog breeding is fast becoming one of the major branches of animal production in Nigeria and a means of poverty alleviation in the country (Otite and Egbunike, 2002). The relationship between dog and man dates back to prehistoric times where the dog was used for hunting. This relationship between man and dog has evolved over the years with the dog now performing numerous roles in the human-companion animal relationship. To date, no extensive research has been reported in the field of canine reproduction in Nigerian. This study is a pioneering investigation and therefore as much information is given in the aspect of canines and companion animals with the hope that this work would form the basis for future research as well as an authoritative point of reference to boost the Nigerian canine industry.

1.1 HISTORY OF THE DOG

History tells us that the oldest animal to be domesticated by man is the dog (*Canis familiaris*). Its origin is believed to date as far back as 40 million years. Drawings and paintings of old times suggest that man's initial contact with the dog was probably about half a million years ago.

It is believed that the social nature of the dog coupled with its scavenging nature was a major attribute which facilitated its domestication.

Domestication is believed to have taken its roots from Europe though there exists no concrete evidence of this. After domestication, next came selective breeding in which specific traits of behaviour and appearance were selected for breeding purposes. Some of such behavioural traits include hunting, guarding as well as herding. Man's occupation in that era suggests that hunting dogs were probably the oldest breeds of dogs to be bred by man. This suggests that the dog's initial role was not necessarily for companionship.

Over 225 registered breeds of dogs are in existence today (Verhoef, 1996), each with its own distinct morphological and genetic characteristics, such as shape, colour, size, temperament and intelligence. Some of the old breeds such as the basenji and saluki are still in existence today. Their origin could be traced as far back as 3,400 and 5000 B.C. respectively. Though these may

show slight variances from the original breeds due to the increase in intensive breeding programmes embarked upon by breeders over the centuries, there are a few breeds of dogs which have not deviated from their original standard. The Italian Greyhound is one of such breeds. It is still possible to find traces of some original breeds in many of today's dog breeds, thus enabling researchers to trace the ancestry of a vast number of present day breeds (Table 1).

Table 1: Dog breeds showing places, dates of origin and probable ancestors.

BREED	PLACE OF ORIGIN	PROBABLE DATE OF ORIGIN	POSSIBLE ANCESTRY
Staffordshire Bull Terrier	Staffordshire England	1800's	Bulldog , local terrier
Leonberger	Germany	1800's	Newfoundland, St. Bernard, Pyrenean Mountain Dog
Doberman Pinscher	Germany	1900's	old shorthaired shepherd, Rottweiler, Black and Tan Terrier and the German Pinscher
Great Dane	Unknown	3000-1121 BC (unverified)	Irish Wolfhound, Old English Mastiff
Australian Cattle Dog	Australia	1800's	Collie, Dalmatian, Black and Tan Kelpie
Dogue de Bordeaux	France	Unknown	Bullmastiff, Bulldog, Tibetan Mastiff, ancient Dogues de Bordeaux of Aquitaine
Bullmastiff	England	1800's	Old English Mastiff, English Bulldog.
Irish Setter	Ireland	1800's	Irish Water Spaniel, Irish Terrier, English Setter, Spaniel, Pointer, Gordon Setter
Miniature Pinscher	Germany	Unknown	Dachshund, Italian Greyhound, Shorthaired German Pinscher

Source: American Kennel Club (AKC, 2011).

1.2 ANCESTRY OF THE DOG

Dogs share the same common ancestor (Miacis) as wolves (*Canis lupus*), coyotes (*Canis lutrans*), foxes (*Vulpes vulpes*), Civet cats (*Civettictis civetta*), hyenas (*Hyaena brunnea*), African hunting dogs (*Lycoon pictus*), South American bush dogs (*Canis aureus*) and the Ihole, all of which belong to the order carnivora and family canidae.

Miacis is believed to have existed about 40 to 50 million years ago (Warren, 2002). Miacis was a short legged, long tailed carnivore which was one of the ancestors of the primitive wolf (*Canis estruscus*) approximately 2 million years ago.

It is widely believed that this primitive wolf was the immediate ancestor of the dog. This is a globally accepted theory as wolves share similar genetic and phenotypic traits as dogs. Wolves do not only move in parks but are social animals and bark like the dog. Wolves and dogs also have similar dentition.

Along with the wolf was a second wolf descendant, the fox. It is an academic belief that the dog and fox are the only descendants of the wolf and that the jackal and coyote broke off from another carnivore, Borophagus. All the above (except the fox) can hybridize to produce fertile offspring, thus confirming the close affinity of members of this genus all of which posses the same chromosomal number of 78 as well as chromosomal size and shape (Robinson, 1999). Pairing of chromosomes is thus possible. (Mendel, 1866). Perhaps this is one major reason why foxes which have 34 chromosomes cannot hybridize with the dog or the wolf. Such may be said about other members of the mammalian class such as the Lion (*Panthera leo*) and the Tiger (*Panthera tigris*) which can interbreed to produce a tigon or liger (depending on the paternal specie) though the tigon being the smaller and more fragile of the two. In previous years, it was assumed that the resulting offspring was sterile in spite of the distinct chromosomal number exhibited by both specie. This was assumed to be due to the disparity in chromosomal size or shape (Otite, 2000). However, recent studies have proved this to be untrue as successful breeding attempts have resulted in fertile hybrids of both specie (Roberts, 1983; Comelab, 2008).

Evidence of such hybrid offspring such as the coyote-dog, coyote-wolf as well as wolf-dog have been spotted in different parts of the world such as Europe and North America. One might not be able to regard the species as perfectly distinct or as Robinson (1999) put it, “the process of

differentiation has not proceeded as far as one might expect". It therefore follows that the dog, wolf, coyote, Jackal and the fox are slightly similar species, gradually drifting apart with the fox farthest away from the rest.

Fig. 1.1: Mammalian hybrids. Above: A wolf Dog Hybrid. Below: A lion Tiger hybrid (Liger) from Novosibirsk zoo

1.3 IMPORTANCE OF THE DOG

In ancient times, dogs were used as status symbols in order to depict power and affluence. For instance, the ancient pharaohs and elites of ancient Egypt kept dogs in order to elevate their status. Equally, wealthy citizens of ancient Greece, Rome and China kept dogs for prestige. Drawings and paintings on ancient relics have been noted to poses adequate proof to this fact. In ancient times, dogs were given human names and a lot of affection was given to them. One could thus infer that dog rearing is not a western attribute as researchers put it because the interaction between dogs and the aristocrats dates back to antiquity. Some scholars in fact attribute dog rearing to Negroid heritage standing firm on their claim that ancient Egyptian civilization precedes all other civilizations and numerous paintings and relics from ancient Egypt have traces of the canine species embedded in them.

Modern day has witnessed the dog playing a vast number of roles unlike in the past were hunting, herding and guarding were works of the day and the basis for dog rearing. Social changes such as rising living standards, of man over time and exposure of individuals to foreign cultures due to the bridge in communication network largely attributed to globalization, have encouraged the growth of the global dog population. Though it is widely believed that the use of the dog as a companion in modern day is truly a western affair, the use of the dog for companionship in the Third World cannot be overemphasized.

Dogs are used as social catalyst and for this reason, dogs posses numerous health benefits (Hooker 2002). They aid in the alleviation of stress and depression in the lonely, helpless and socially depressed. In some instances, they play the part of a deceased friend or relative (Bustad and Hines 1983; Lago *et al.,* 1985). A study of bereaved women showed that women with strong attachment to their pets reported less grief and deterioration of health following the death of a spouse than those without pets (Bolin, 1987). Stallones *et al*. (1988) associated pet ownership with reduced depression in elderly people. Dogs are therefore buffers against stress and grief (Serpell, 1995). Excessive stress has been known to lead to mental and physical ill health such as hypertension in elderly people. It has now been proven that regular walks with companion animals help influence proper breathing and heart beat coordination and in turn reduce the risk of high blood pressure and other heart related diseases. This in turn ensures proper blood flow within the body. Researchers have shown over the years that people who possess companion

animals especially dogs, show less risks of heart problems. Katcher, 1981 also observed that reduced heart rate and blood pressure could be achieved through simple stroking or interaction with pets. Anderson *et al*. (1992) also observed reduced rate of coronary heart disease among pet owners. Dogs make an excellent source of recreation for the handicapped, business and aged class. Siegel (1990) also observed that people with pets had less doctor contacts than those without pets. In regards to AIDS, Siegel *et al*. (1999) observed higher depression in victims without pets than those with pets.

All the above qualities have brought about the use of dogs in clinics in some countries for their use in pet therapy also called Pet Facilitated Therapy (PFT). To date, over 50% of nursing homes and other health facilities employ the services of the dog as some form of therapy (Olsten, 1985). Presently, several pet visiting schemes exist globally. The Pro-Dogs Active Therapy of Great Britain is one of such examples.

Researchers have put forward that children are positively affected by the presence of animals in the home. In fact, the presence of companion animals in households aids the development of responsibility traits in infants. Looking after animals enhances independence, reliability as well as boosts the gradual build up of adult qualities in young children. Nursing bitches with puppies could be a child's first introduction to the good side of parenthood and family life. Most dog breeds are able to tolerate children in the house as well as their problems; this is another advantage companion animals have over human-human relationships. The non-selfish nature of dogs, their positive response to correction, as well as their non-hostile and social nature helps break similar traits in infants due to the anthropomorphic role they play. Communicating with companion animals in times of despair also has its advantages as animals (excluding parrots) can keep secretes while humans cannot.

As guards, dogs repel buglers, petty thieves and intruders who are put off by their noisy barking. Over the years, dogs have proven to be more reliable than most other forms of security in the 3rd world countries as the use of sophisticated scientific devices are not predominant due to the ailing economies associated with most 3rd world countries. The escalating occurrence of crime and ineffective police response to address this issue has been matched with the massive increase in the population of large dog breeds such as the Rottweiler, German Shepherd, Doberman, Rhodesian Ridgeback and for the wealthy, the Great Dane and Mastiff.

Dogs play a major role in police and security work. Their unrivalled ability to detect hard drugs, stolen objects, apprehend criminals, guard property and bank premises makes them highly favoured for the job.

In certain third world countries, dogs are used as table meat. In such countries, dogs are highly appreciated as a delicacy and are sold off after they have outlived their usefulness. In local Nigerian restaurants they are consumed with local alcohol and other drinks and their body parts given nick names such as the gear box (heart), accelerator (feet), floater (lungs) etc.

In various villages around the world such as in Africa and Asia, dogs hold a superstitious high position in witchcraft and their service is rivalled only by the domestic cat (*Felis domestica*). Most people killed for witchcraft have been observed to show affection for one or more companion animal in which case the dog or cat is usually the choice of preference. There also exists a superstitious belief that dogs see spirits, witches, angels and other fictitious beings.

Table 2: Nutritional value of dog meat (per 100g)

Energy	1,096 kJ (262 kcal)
Carbohydrates	0.1 g
Dietary fibre	0 g
Fat	20.2 g
Protein	19 g
Water	60.1 g
Vitamin A	equiv. 3.6 µg (0%)
Thiamine (Vit. B_1)	0.12 mg (9%)
Riboflavin (Vit. B_2)	0.18 mg (12%)
Niacin (Vit. B_3)	1.9 mg (13%)
Vitamin C	3 mg (5%)
Calcium	8 mg (1%)
Iron	2.8 mg (22%)
Phosphorus	168 mg (24%)
Potassium	270 mg (6%)
Sodium	72 mg (3%)
Ash	0.8 g

Source: Yong-Geun (1999)

1.4 The Nigerian Dog Trade Industry

Due to demographic and social changes over the years, the dog trade industry has achieved tremendous growth and success with a net profit at an all time high. The ever growing global dog population has resulted in about 10.5 million pet dogs in the U.K alone (Murray *et al*., 2010), a figure which outnumbers human populations in countries such as Eritrea, Israel, Kuwait and Panama with human populations of about 5.2, 7.6, 3.1 and 3.5, million respectively (PRB, 2010). Today, registered breeds sell at costs ranging from $250 to as much as $50,000.

In Nigeria, the dog trade industry has contributed immensely to the reduction in unemployment caused by an ever growing human population as well as global recession. Today, numerous companies constitute the global pet food industry. The overall pet food market is currently estimated at US$80 billion with the dog and cat food industry accounting for 55% of this (Taylor, 2011). Grooming establishments, vet care, accessories, boarding and training as well as importation and exportation of dogs also show signs of prospects in this growing industry.

The dog industry in the tropical third world, though still crawling, should never be overlooked as it has a lot in stock for the future. Most third world countries possess small boarding facilities accommodating a rough average of 50 dogs. The existence of a handicapped feed industry greatly attributed to the unavailability of Animal Scientists/nutritionists due to lack of enforced government laws has left the feed industry crippled by veterinarians delving into a field which I consider alien to their profession. The gradual trend of change from dictatorship leaderships to democratic governments in most third world countries has also had a positive impact on the influx of expatriates and subsequently the importation of exotic dogs due to the stability that accompanies it.

Artificial insemination in dogs, which is one way of reducing the high risk of disease transfer (of which dogs are highly vulnerable to, especially in the tropics) as well as reducing the amount of time and energy expended while mating aggressive or timid dogs (which could be cumbersome), can still be described as a virgin area of specialisation in Nigeria unlike the advanced world. Exotic dog breeds with the exception of the Rhodesian Ridgeback and the South African Boerboel are not of African origin and hence their population in the country is still puny when compared to the population of indigenous dog breeds in the country. Due to this small

population, locating distant related strains for the preservation of extant genetic diversity in order to create heterosis, could be very cumbersome and hence, the employment of semen preservation techniques for use in artificial insemination would definitely come in as a useful tool as the burden involved in transporting stud dogs and bitches across borders or long distances to their designated place of breeding would be significantly reduced.

To date, data on dog semen, quality after dilution and preservation are scanty in Nigeria. Semen extension and transportation requires great expertise as dog spermatozoa have been reported to have a reduced post storage fertility period (England and Lofstedt, 2000). Therefore, there is a need for thorough research to be carried out on canine semen extension/preservation as this would serve as a great boost not just to the Nigerian kennel industry but also to other livestock enterprises.

1.5 OBJECTIVE OF STUDY

The broad objective of this study is to investigate the storage ability of semen extenders as well as to propose a method of revitalizing post thawed cryopreserved canine Spermatozoa. The specific objectives of this study are:

1/. To look carefully into the ability of various semen extenders to maintain semen quality over a period of time, thus proposing an adequate extender for preserving canine semen at different preservation temperatures.

2/. To use already established methods of estimating the fertilizing ability of spermatozoa to determine the maximum viability period of preserved canine semen prior to insemination.

3/. To study the effects of cryopreservation of extended canine semen taking into consideration the changes that occur as a result of the freezing and thawing process.

4/ To look into the ability of Chloroquine Phosphate as a means of rejuvenating post thawed cryopreserved canine spermatozoa.

1.6 JUSTIFICATION

The awareness of the huge profits associated with dog breeding is on the increase in the country and as a result, there is an increase in the number of dog breeders in the country.

The cost difference between indigenous dogs and exotic dog breeds has resulted in an increased demand for exotic dogs.

The high level of importation of exotic dogs into the country has financial implications on the nation's staggering economy. Quarantine laws are hardly enforced and records of dogs brought into the country are not properly documented. Besides this having an undocumented effect on the nation's economy, it is also a source of disease entry into the country. Hence, the application of more advanced methods of reproduction such as semen extension and preservation as well as artificial insemination would come in handy in reducing this demand for importation of exotic dog breeds.

Data on semen cryopreservation only tokenistically covers companion animals of which the dog belongs. As a result of this, there exist little information on canine semen extension and cryopreservation in Nigeria. Majority of the works available were performed under temperate conditions and hence might not apply in the tropics.

Due to the poor training of border staff in the country, imported semen is left unattended for hours and hence semen viability is reduced resulting in financial loss to the importer. There thus lies a need to investigate the use of drugs such as Chloroquine Phosphate as a means of boosting the fertilizing ability of cryopreserved canine spermatozoa.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 REPRODUCTION IN THE DOG

2.1.1.1 Hormones of Reproduction

Dogs are monestrous and hence ovulate every 6-12 months. This has huge financial implications on the pockets of breeders as any failed mating could result in breeders having to go for as long as a whole year without producing puppies resulting in loss of financial revenue. This is one of the greatest hitches associated with dog breeding. For a thorough breeding program especially one which relies heavily on the use of more advanced methods of reproduction, such as artificial insemination, cryopreserved semen and embryo transfer, a thorough understanding of the hormones of reproduction is of vital importance as this could make the difference between a successful and unsuccessful breeding programme. Differentiation between the species is also of invaluable importance as variation between specie is also very common. Thorough knowledge of the hormones of reproduction in dogs and bitches are a priceless tool to tell us the exact time to inseminate animals as well as sustaining pregnancy after a successful mating has been performed.

2.1.1.2 Releasing Hormones: Majority of the hormones that control reproductive function in the dog are controlled by releasing hormones produced by the hypothalamus.

These hormones act on the anterior pituitary gland to bring about the release of specific hormones. In most cases, these hormones are interlinked. Thus, a deficiency of one could result in an increase or decrease of the other thus making diagnosis a little difficult in some cases.

Due to the ability of most releasing hormones to be rapidly metabolized by the liver, the body has a more specialized way of delivering releasing hormones to the anterior pituitary. This system of transportation is known as the hypothalamic-pituitary portal system (Hill *et al*., 2008).

2.1.1.3 Gonadotropin Releasing Hormone (GNRH): The chief releasing hormone of importance in reproduction is gonadotropin releasing hormone (GNRH). GNRH is a decapeptide molecule released by the hypothalamus in pulses or rhythms. Its release is usually more frequent

as the dog approaches oestrus. One particular attribute of this releasing hormone is that it has the same structure in all domestic animals and hence it is readily produced and can easily be used across various animal species.

GNRH causes the release of luteinizing hormone (LH) and to a lesser degree follicle stimulating hormone (FSH). In earlier times, it was believed to be the chief releasing hormone for FSH in dogs but recent studies have suggested another releasing hormone for FSH as sometimes when the LH level is meant to be low for example in the case of a functional corps luteum (corpus luteum produces progesterone which should technically result in a reduction of LH and FSH), the level of serum FSH is surprisingly high. Also in cases of high concentration of GNRH, the LH increase is usually higher than the FSH increase. It is for this reason that GNRH is sometimes referred to as LHRH. Though an increased level of LH brings about ovulation and hence GNRH can be used in synchronizing oestrus in some animals, its use in oestrus synchronization in dogs is not always effective. This is usually the case as well in single ovulators as this is highly dependent on the stage of follicular development. Other releasing hormones produced by the hypothalamus include, Growth Hormone Releasing Hormone which causes the release of Growth Hormone (GH) and Thyroid Releasing Hormone (TRH) which brings about the release of Thyroid hormone and prolactin.

2.1.1.4 Follicle Stimulating Hormone (FSH): FSH is a glycoprotein hormone produced in the anterior pituitary gland. Its function is to bring about maturation of follicles and works in hand with LH. This it does by stimulating the primary follicles to develop into larger secondary follicles. As a glycoprotein hormone, it is a huge molecule and hence unstable. As a result of its highly unstable nature, it is usually stored in freeze dried state.

The structure of the glycoprotein hormones differs from specie to specie and hence the specie specific assays are produced. In some cases however, these assays can work across a few species.

In males, FSH influences the production of Androgen Binding Protein (ABP) which binds testosterone; thus making it available in high concentrations in the seminiferous tubules. This high concentration of ABP is needed for spermatogenesis. Since the testes produce a large amount of oestrogen, FSH is the hormone responsible for controlling oestrogen synthesis. Hence in cases cryptorchidism, a large amount of oestrogens can be observed through hormonal assay.

2.1.1.5 Luteinizing hormone (LH): LH works in conjunction with FSH. FSH induces receptor sites for LH thus increasing the effects of LH on the follicles. LH assists in follicular maturation. As the follicles mature, they produce estrogens which in turn send messages to the hypothalamus. This effect of estrogens on the hypothalamus stimulates an increase in the pulsative secretion of GNRH which in turn results in an increase in LH resulting in the LH surge. It is this LH surge that brings about the ovulation of follicles in dogs.

After maturation, the LH level drops and LH is then produced in small quantities. It is this basal pulses of LH that bring about the formation of the corpus luteum as well as the maintenance of pregnancy. The later is understandable as progesterone aids in maintaining pregnancy and hence since progesterone concentration is usually inversely related to the concentration of serum LH, it thus follows that if LH is low then progesterone concentration would be high.

In males, LH is responsible for the production of testosterone as it influences the leydig cells in the testicles to produce testosterone (Lofstedt, 2004). Hence when referring to male dogs, LH is sometimes referred to as interstitial cell stimulating hormone (ICSH).

2.1.1.6 Inhibin: As the follicles attain maturity, they produce a hormone known as inhibin. Inhibin suppresses the secretion of FSH due to the negative feedback it has on the hypothalamus. This hormone prevents the maturation of other follicles thus preventing multiple ovulations, explaining why animals such as cattle usually have a single follicle ovulating at a time and hence give birth to one offspring (Lofstedt, 2004). However, in the dog, the production of inhibin by a single follicle is usually insufficient to suppress the maturation of other follicles hence we refer to the cumulative effect of inhibin with regards to regulating the number of ovulated follicles. In some cases this can range from 1 to over 10 follicles. It is for this reason that fertility tests in bitches might not be a conclusive method of estimating a studs fertilizing potential.

2.1.1.7 Androgens: There are numerous androgens produced by male dogs. The androgen of cardinal importance in reproduction is testosterone. Testosterone has numerous functions in both male and female dogs. Included in these functions are masculinisation, libido, aggression, growth stimulation, maintenance of spermatogenesis and the function of the assessory sex glands. Most male animals do not have receptor sites for testosterone hence testosterone must be converted to dihydroxy testosterone (DHT) by the enzyme 5 α reductase. This enzyme is very important in

reproduction in the dog as some dogs lack this enzyme and consequently would be insensitive to already present testosterone hence the attributes of testosterone would be absent making the animal appear phenotypically female (Radovick and MacGillivray, 2003). Androgens are also produced in bitches and in clinical practice, they can also be used to postpone oestrus as well as reverse feminism (Noakes *et al.*, 2001).

2.1.1.8 Estrogens: Like the androgens, estrogens are sex steroids, relatively small and stable and easy to manufacture. They are important in producing female sexual behaviour in bitches. Estrogens are responsible for hypertrophy (increase in tissue size as a result of the enlargement of existing cells) of various organs associated with reproduction such as the vulva, mammary glands, cervix and vaginal mucosa.

Progesterone: Progesterone is produced by the corpus luteum, placenta and to a lesser degree the adrenal gland. It has numerous functions which include the maintenance of pregnancy during gestation. This is achieved through the negative effect it has on GNRH which suppresses the release of LH and consequently preventing further follicular growth, maturation and ovulation. It also brings about oestrus behaviour in bitches by stimulating behavioural centres in the brain. In the bitch, during pregnancy the corpus luteum produces most of the progesterone required for maintenance of pregnancy (Lofstedt, 2004) and hence the removal of the ovaries during pregnancy usually results in abortion. This is not the case in cattle and humans as the bulk of progesterone during pregnancy is of placental origin.

2.1.1.9 Prolactin: Besides being responsible for galactogenesis in bitches, prolactin plays an important role in maintaining pregnancy as it plays a defined role in the maintenance of the corpus luteum (Onclin, and Verstegen, 1997). This has been clinically proven as prolactin is negatively dependent on another hormone dopamine. It is for this reason that in cases of agalactia, dopamine suppressants such as (phenothiazine tranquilizers) are administered (Riviere and Papich (2009). Further proof to the role or prolactin in maintaining pregnancy has been demonstrated by Cetin *et al.* (2010) who showed that the administration of dopamine like compounds (such as the ergot alkaloids) resulted in abortion in bitches**.**

2.1.1.10 Oxytocin: Oxytocin is produced mainly in the hypothalamus (in an episodic fashion) and partly by the corpus luteum during the second half of the oestrus cycle. Oxytocin hastens

parturition through its direct effect on the myometrium by releasing prostaglandins F2 and E2 from the endometrium. Prostaglandins have a great effect on luteolysis. Oxytocin also play an important role in milk let down (not agalactia) as it causes contraction of the mammary gland. Hence for pregnancy to be maintained after a successful insemination, the level or oxytocin should be low in order for the bitch to carry on with the gestation process.

2.1.1.11 The Placental Gonadotropins: Of chief importance are HCG (Human Chorionic Gonadotropin) and ECG (Equine Chorionic Gonadotropin also referred to as PMS (Pregnant Mare Serum). Though the above hormones are not of canine origin, their importance in dog breeding makes them worthy of mention due to their use in oestrus synchronization.

ECG has FSH like properties and is thus a useful hormone for stimulating follicular development. It can be used in breeding programs that depend on follicular stimulation such as multiple ovulation and embryo transfer. However, the use of ECG is limited as its high concentration of sialic acid makes it difficult to control its follicular stimulating properties (Loftdest, 2004).

Although HCG originates from humans and primates, HCG has LH like properties and hence comes in handy when the induction of ovulation in bitches is required (Schaer, 2010). The drug Pg600 (produced by Intervet International), initially intended for porcine use has recently been used by numerous dog breeders to stimulate fertile oestrous. Pg600 is a combination of HCG and ECG.

2.1.1.12 Prostaglandin: The most important prostaglandins are the E and F series. Prostaglandins play a major role in Canine reproduction as they cause luteolysis of the corpus luteum, preparing the bitch for parturition. Prostaglandins also play an important role in the process of ovulation as they affect the release of GNRH in bitches (Lofstedt, 2004).

2.1.2 The reproductive cycle of the dog:

Dogs attain puberty (when the dog is able to produce a fertile ejaculate) at the average age of 9 months. However, this may vary between and among breeds due to the vast disparity in size (Pineda and Dooley, 2003) associated with various breeds. Smaller breeds usually attain puberty at an earlier age (Pineda, and Dooley, 2003) in some cases as early as 6 months) while the larger

breed can take as long as 18 months to attain puberty. Before puberty can be attained, the dog must have attained a minimum of 60-70% of its matured body weight (Lofstedt 2004). This implies that the attainance of puberty in dogs is largely dependent on the dog's nutritional status. Not all puppies are born with their testis in their scrotum.

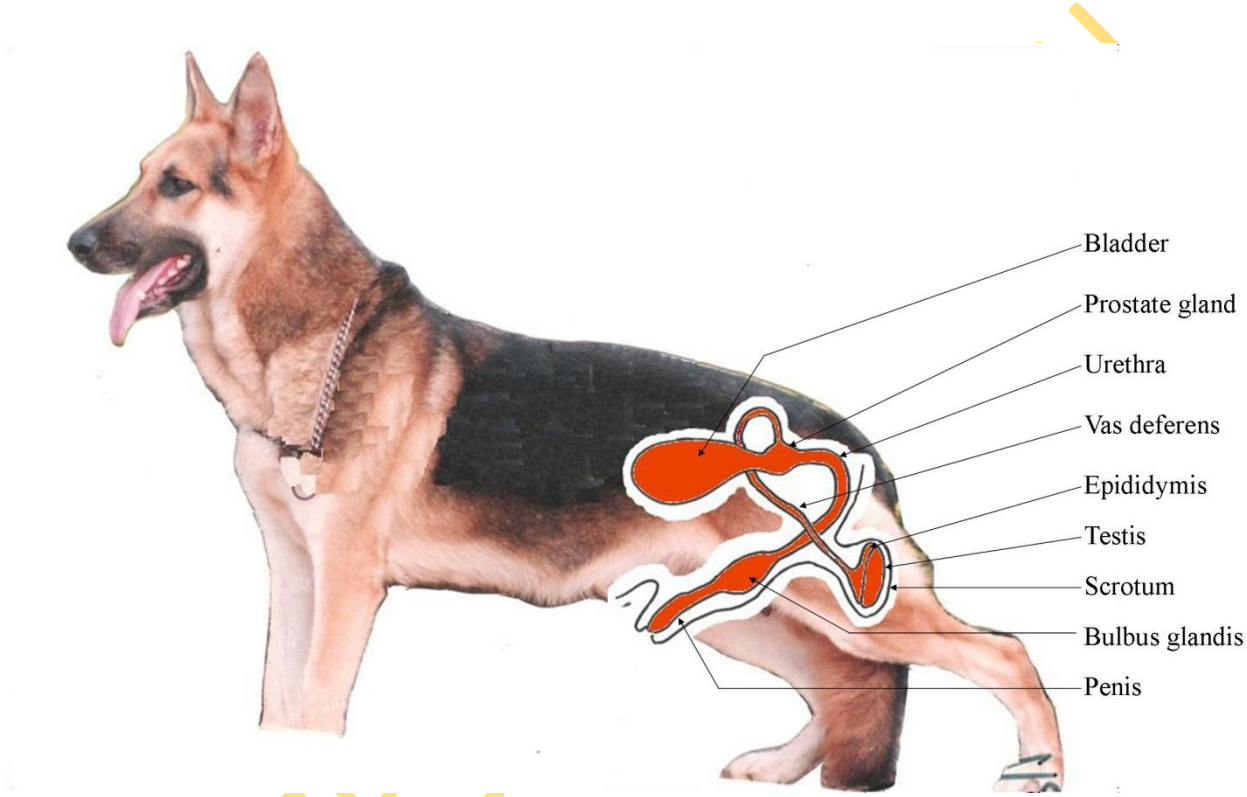


Fig. 2.1: The reproductive tract of the dog.

2.1.2.1 Characteristics of dog semen: Canine semen is made up of the spermatozoa and the seminal plasma. If left to stand, semen divides into two portions, the spermatozoa at the bottom and the watery transparent portion on top. Various ranges of semen volume have been giving for the dog. Bartlett (1962) gave the average volume of semen to be about 12ml, while Otite and Egbunike (2000) recorded an average semen volume of 16.82ml with a range of 2.45 to 50ml. This total ejaculate volume is largely dependent on the body weight of the dog (Dubiel, 2004), as well as the type of diet (Otite and Egbunike, 2001). Canine semen is greyish to milky in colour with a characteristic pungent smell and a pH ranging from 6.4-6.8 immediately after collection Barber (2010). However, this value drops with time due to the accumulation of lactic acid as a

result of the metabolic activities of the actively motile spermatozoa (Norman *et al.*, 1958). A complete ejaculate from the dog is made up of three fractions. These fractions are voided in intervals ranging from 10-20seconds (Harrop, 1955). This also varies among individual dogs. The different fractions can be separately collected by switching the collection bottles in between fractions. This makes it possible to perform and artificial insemination using only the sperm rich portion.

2.1.2.2 First Fraction: The first fraction is voided during intromission and is devoid of spermatozoa. This absence of spermatozoa gives this fraction its clear watery appearance. Its volume ranges from 0.25-5ml (Harrop, 1960). It is the secretion of the urethral mucous glands (glands of littre). This fraction also serves to wash the vaginal tract as well as lubricate the tract thus serving to prevent bruising or trans-contamination of disease due to blood contact during intromission. The first fraction has a pH of 6.73 and its voidance could last from 30-50seconds. This portion is the most contaminated with bacteria (Goericke-Pesch *et al.* 2011).

2.1.2.3 Second Fraction: This is the sperm rich portion of the ejaculate (England *et al.*, 1990) and is milky in colour giving the semen its characteristic milky appearance. It is more viscous in consistency than the other two fractions. This is the chief produce and secretion of the testes. Even though in most artificial inseminations all the three fractions are used, this is indeed the chief fraction of interest in artificial insemination (Thomassen & Farstadt, 2009). This fraction is the most acidic and has a pH of 6.10. The total volume of this discharge is between 0.5 and 3.5ml (Harrop, 1960) and could last as long as 90 seconds to be voided.

2.1.2.4 Third Fraction: This fraction is of prostatic origin and is chiefly responsible for the difference in volume as well as the duration of the tie associated with copulation in this specie. Farstad, (2010) observed that this fraction makes up for as much as 95% of the total ejaculate. Its voidance could last as long as 35minutes. The volume of this portion could be as much as 40ml (Johnson *et al.*, 2001). This fraction is what gives semen its characteristic smell. The third fraction has a pH of 7.20 and its alkaline nature is responsible for the increase in mass activity of the spermatozoa. This fraction serves to activate the spermatozoa. This can easily be understood as canine spermatozoal motility increases with alkalinity (Carr *et al.*, 1985).

2.1.2.5 Concentration: The appearance of Canine semen is largely dependent on the concentration (Bartlett, 1962). The higher the concentration, the greater the opacity. Different concentrations have been giving by numerous researches in regards to the concentration of canine semen. Oettle (1993) and Linde-Forsberg (1991) observed a range of 50 x106 up to 1575 x 106 Spermatozoa per ejaculate. This is also dependent on age, testicular weight, sexual activity and dog size (Amann, 1986). Blackledge, (1958) observed concentrations as high as 600 million per millilitre, while 100x 10^6/ml was observed by Schafer-Somi *et al.* (2006).

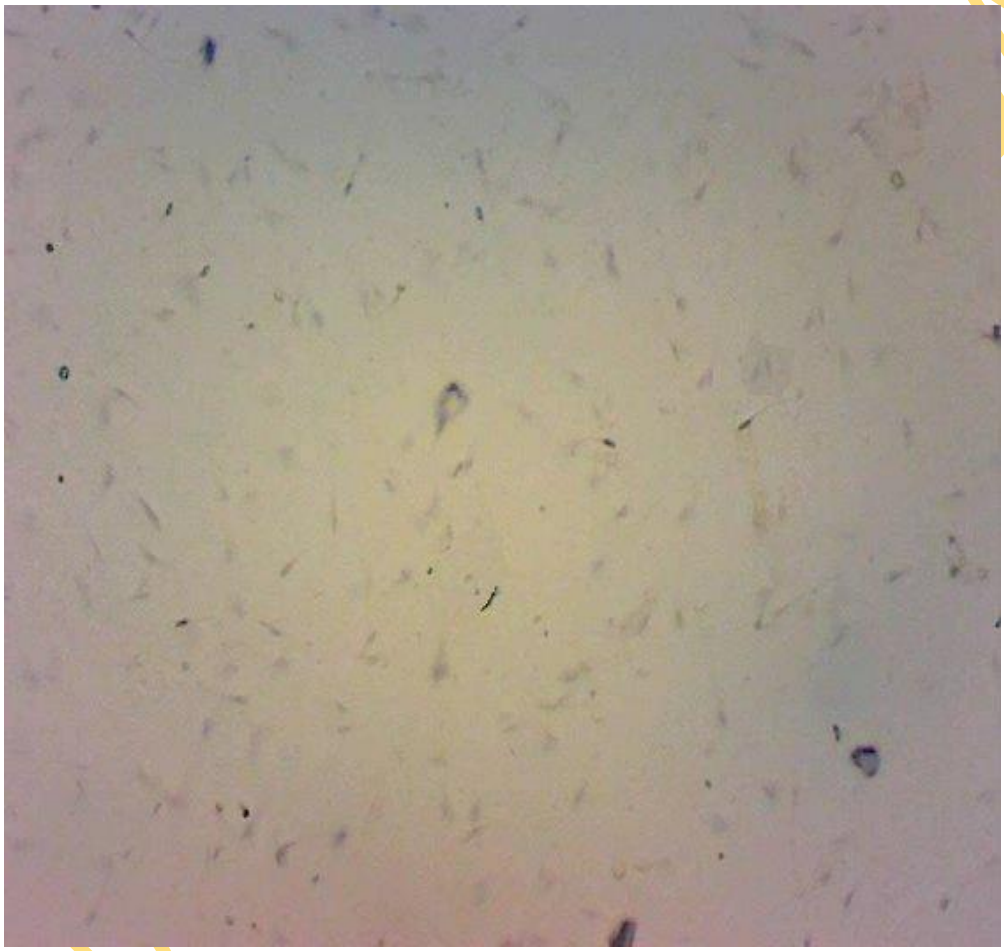


Fig. 2.2: Canine spermatozoa under the microscope (X40)

It appears though that these results are incohesive because many researchers use only the sperm rich portion and not the complete ejaculate which would have been diluted by the first and third fraction.

2.1.2.6 Mass Activity: Good quality canine semen should have a mass activity not less than 50%. Microscopic examination of canine semen shows that canine semen is of extremely high activity and has a rippling motion quite distinct from the wavelike motion associated with bovine semen. However, this is highly dependent on the volume of semen (Otite 2000). High Mass activity gives spermatozoa the ability to penetrate the mucus membrane (Suarez and Osman, 1987) and is required by sperm to progress towards the oocyte. This high mass activity aids in sperm capacitation and on reaching the oocytes, capacitated sperm dissolve the zona pellucida. Hyperactivation is required to penetrate this cell wall. Stauss *et al.* (1995) observed that when hyperactivation was blocked in capacitated sperm, spermatozoa bound by their acrosome to the zona pellucida, they were unable to penetrate it.

2.1.2.7 Progressive motility: In canine semen analysis, only the spermatozoa moving in a progressive forward direction are of importance as this ensures that the head of the spermatozoon comes in direct contact with the egg to begin the onset of fertilization. Although normal dog semen contains at least 70% of progressively motile spermatozoa (Feldman & Nelson, 1996; Günzel-Apel, 1994), it is recommended that this value should not fall below 60% (Lofstedt, 2004). Other researchers have suggested a lower recommendation of 50% (Farstad, 2010) for frozen thawed semen. Over the years numerous researchers have stuck to this 60% minimum value as standard for top quality semen. In spite of this, recent research has indicated a much lower value. Thomassen *et al.* (2001) classified good quality semen as having over 50% motility while poor quality semen has motility below 50%. Conception rates of 40% have also been cited by Froman *et al.* (1984) from inseminations carried out using semen with progressive motility as low as 35-40%. With the advent of more modern methods of breeding, this value is constantly on the decrease. An example of such is Intra Cytoplasmic Sperm Injection (ICSI) which eliminates the need for progressive motility (Elder and Dale, 2011) as the spermatozoon is injected directly into the cytoplasm of the ovum.

2.1.2.8 Morphology: All the different types of abnormalities of mammalian spermatozoa can be observed in dog semen. These include detached head, double head (bi cephaly), micro cephaly, macrocephaly, double tail, bent tail, coiled tails and tail stump. Arthur (1977) observed that the detached head, coiled tail and bent tail were the most common types of abnormalities associated with canine spermatozoa. Initially it was accepted that the percentage abnormalities

of good semen should not exceed 30% (Günzel-Apel, 1994). However, more resent works have suggested a minimum of value 40% abnormality (Blendinger, 2007).

2.1.2.9 Structure of Canine Spermatozoa: The ideal spermatozoon is an elongated tadpole like cell made up of the head, neck and the tail piece. The spermatozoon is simply a basic restructuring of the general cell in which the nucleus forms the head; the centrioles rearrange to form the neck while the mitochondria form the tail piece.

2.1.2.10 The head: This contains the nucleus and the cytoplasm. The Nucleus contains the genetic material of the sperm cell. There are 78 chromosomes present in the nucleus (Lindblad-Toh *et al.*, 2005). Not all domestic dogs have 78 chromosomes. There exists the XXY male dog (Clough *et al.*, 1970) which has 79 Chromosomes rendering the bearer sterile due to the absence of spermatozoa in the semen.

The head is covered by a flattened cap called the acrosome. The acrosome is very important in natural and artificial mating as it contains a range of glycoproteins and hydrophilic enzymes (principally hyalurinidae) which help dissolve the zona pellucida (Thick outer membrane surrounding the mature ovum) in order for contents of the nucleus to flow into the egg.

2.1.2.11 The neck: The neck of the spermatozoon is a short piece serving as a connector between the head and the tail. It contains a lot of the cytoplasm of the cell as well as traces of the centrioles.

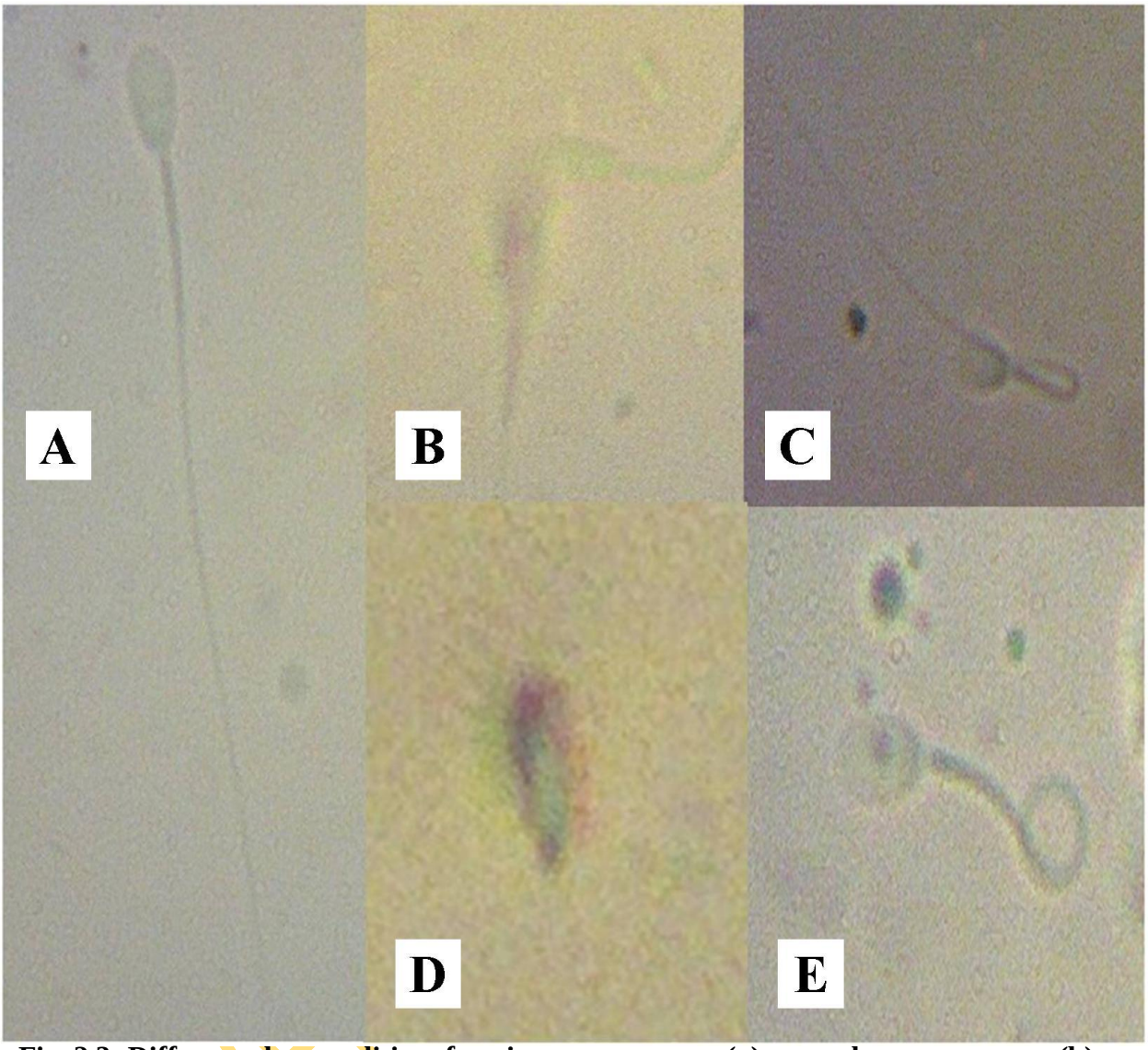


Fig. 2.3: Different abnormalities of canine spermatozoa. (a) normal spermatozoon, (b) normal spermatozoon with DNA intact, (c) bent tail, (d) detached head (e) coiled tail.

2.1.2.12 The tail: The tail contains the mitochondria which provide the energy required for movement and is thus the power house of the cell. The tail is an elongated flagella like structure made up of 3 parts. These are the middle piece which contains the axoneme and mitochondria, the principal piece (containing the axoneme and nine coarse fibres from the middle piece) and the end piece which contains the axoneme alone.

2.1.2.13 Seminal Plasma: This is the second component of semen and is the fluid portion. It contains nutrients which help nourish and maintain the spermatozoa. The seminal plasma also has buffering abilities to help maintain the pH of the medium. In semen analysis, the quality of

the seminal plasma gives a valid indication of the survival ability of the spermatozoa. In canines, the sperm concentration is usually the same if only the sperm rich (second fraction) fraction of the ejaculate is analysed, hence the total ejaculate volume which is made up mostly of the seminal fluid would be an indication of the survival time of the collected spermatozoa.

2.1.2.14 Survival time of Canine spermatozoa: Canine spermatozoa has a longer life span in the reproductive tract of the bitch as the fluids produced by the bitch help nourish the spermatozoa. Doak *et al.* (1961) recorded a maximum of 11 days after copulation for canine spermatozoa in the reproductive tract of the bitch. This figure is reduced to hours when semen is left to stand at room temperature. Otite (2000) recorded a maximum time of 14.11hrs for German Shepherd Semen left to stand at room temperature. These results fall far below those of Bederk (1933) who observed a maximum life span of 21hours for canine semen left to stand at room temperature. These conflicting results are understandable as the researcher (Bederk, 1933) worked under temperate conditions and not only does the tropical climate aid the growth of bacteria, it does increases the activity of spermatozoa. Hence, works done under temperate conditions would exhibit reduced spermatozoa activity resulting in an extended lifespan of the spermatozoa due to reduction in metabolic activity. Such a reduction in metabolic activity would in turn reduce the amount of acid build up in the medium. Canine Spermatozoa are pH specific (Crusco Dos Santos *et al.*, 1999).

2.1.3. Factors affecting semen quality

Certain factors have been associated with poor semen quality in dogs. These include the following:

2.1.3.1 Age: The age of the dog used for breeding is vital. Though some dogs produce fertile ejaculate as early as 6 months, they reach peak production at the age of 3yrs after which semen quality goes on the decline. Rijsselaere *et al.* (2007) also observed that the percentage of normal spermatozoa declined with age. It is advisable to preserve semen from proven studs while the dogs are in their prime.

2.1.3.2 Breed: Some breeds of dogs have been associated with low fertility. Dahlbom *et al.* (1995) observed low fertility in Irish Wolfhounds. Rijsselaere *et al.* (2007) observed an increase

in total sperm output with increasing body weight. Dahlbom *et al.* (1995) also observed that bodyweight correlated significantly with total sperm count in dogs.

2.1.3.3 Nutrition: Dogs are omnivores (Hirakawa, 1998), and can thus survive on 100% plant protein as long as their nutritional needs are met. The nutritional status of the dog plays a major role on the quality of the dog's semen. Dogs however can only obtain a small amount of their dietary mineral requirement from plant products (Schugel, 1982). Schugel (1982) also traced zinc deficiency in dogs to commercial dog foods which contain a higher proportion of plant products. Zinc deficiency has also been linked with infertility (Jameson, 1976). Otite and Egbunike (2001) observed a higher body weight in dogs fed on table scraps. Excessive weight elicits hip dysplasia (Robinson, 1992) which hinders natural mating in male dogs. NERI (1999), also associated erectile dysfunction with excessive weight. This is understood as an erection is brought about by blood flow to the penis. The heart being the pumping organ (due to its rhythmic pumping) is weakened by fat depositions in the arteries and hence erectile dysfunction and low libido could occur in overweight animals. Igboeli (1974) observed that the composition of feeds has an effect on the colour of semen. Teitelbaum and Grant (1956) observed that sperm counts increased in dogs when they were starved. However, starvation in form of food withdrawal should not be confused with malnutrition which results in poor semen quality.

Dogs are highly susceptible to micotoxins. Besides the hazardous effects of micotoxins on the liver and blood stream, micotoxins have adverse effects on mitochondria permeability (Hsieh, 1987). This inhibits energy production and hence explains why it affects survival time of spermatozoa.

Micotoxins would thus reduce libido and semen quality in male dogs. Certain feedstuffs have been identified as carriers of micotoxins. One of such feed stuffs is Maize (*Zea mays*) known globally for its content of the fungus *Aspergillus flavos* which produces a toxin known as aflatoxin. Unlike a variety of other toxins such as mimosine, aflatoxin is heat stable and grows on moist maize and other cereals. Maize with less than 10% moisture content should only be used in dog food. Cyanide, found in some plants such as cassava (HHS, 2006) has also been observed to cause a 50% reduction in respiratory rate when included in the diets of rats (Matijak-Schaper and Alarie 1982). The US Department for Health and Human Services (HHS, 2006) also observed that dogs were the most sensitive of all species and also that dogs which ingested as

little as 1.04mg CN–/kg/day as sodium cyanide in a rice diet or as the equivalent cassava diet, showed a reduction in the spermatogenic cycle, testicular germ cell sloughing and degeneration, and occasional abnormal cells.

Hirakawa (1998) also observed that dogs fed excessive amounts of chocolates died as a result of theobromine present in chocolate. Onions have also been known to result in anaemia in dogs (Kellems and Church (1998).

2.1.3.4 Excessive weight: Excessive weight resulting from over caloric intake has been known to affect semen quality. Excessive weight has an effect on the heart causing blocked arteries, resulting in heart failure and erectile disfunction as well as the production of poor quality semen (NERI, 1999).

Excessive weight is a function of weight and height (body length in the case of four legged mammals) otherwise referred to as Body Mass Index (BMI). Excessive weight also gives rise to skeletal problems resulting in difficulty in standing during mating which is however breed specific. The American Kennel Club (AKC) and the Canadian Kennel Club (CKC) have laid down specifications on the weight standard of different breeds. These standards vary slightly from organization to organization. While exotic breeds raised in the tropics are expected to have a reduced matured body weight due to the effects of temperature on performance, there is no standard laid down for dogs in Nigeria as the country does not have a recognized Kennel Club.

2.1.3.5 Scrotal Thickness: For spermatogenesis to occur, the temperature of the testis should be below the temperature of the abdominal cavity (Moore and Quick, 1924; Hammond & Asdell, 1927; Esser, 1932). This is achieved through evaporation from the scrotal skin, a counter-current heat exchange mechanism in the spermatic cord (pampiniform plexus) and involuntary muscular control affecting both the thickness and surface area of the scrotum (tunica dartos muscle) as well as the closeness of contact of the testes to the body wall by the cremester muscle of the spermatic cord (Herman *et al.,* 1994). Olar *et al.* (1981) attributed 79% of the variation in stabilized daily sperm output to scrotal thickness.

2.1.3.6 Testis size/diameter: A fully functional testis should have proper conformation. Amman (1986) observed a positive correlation between testis size and sperm output. There is a

great difference between testis weight and testis diameter hence the testis with largest diameter might not necessarily have the highest sperm output.

2.1.3.7 Frequency of collection:, Chansilpa and Orankanok (1999) suggested a 2 to 3 days interval for semen collection in dogs. Dogs can be used at stud 2 to 3 times weekly. Excessive use of the dog might reduce fertility rate as this adversely affects ejaculate volume (England 1999). Harrop (1960) also observed that too frequent ejaculations results in depletion of the sperm reserves and hence the number of premature spermatozoa increases. These results were also supported by Barber (2010) where she recorded a gradual depletion of the sperm reserves after 4 or more ejaculates were obtained in a few days. However, these results affect the concentration of spermatozoa but not the percentage progressive motility (Barber, 2010). Dogs which are overused and suffer loss of sperm quality usually have their quality return to normal after a 24-72hr rest. Barber (2010) deposed that there exist a higher percentage of morphologically abnormal spermatozoa due to the presence of aged spermatozoa from epididymal storage which usually is the case in dog which are not used at stud over a long period of time.

2.1.3.8 Temperature: An increase in temperature would affect the dogs desire to mate due to the increase in respiration rate as a result of the dog trying to regulate its own body temperature. This makes semen collection arduous and affects the general collection process and semen output. Collected semen has a shorter life span when left to stand at high temperatures (Bartlett, 1962) as this increases spermatozoal activity. Such an increase in spermatozoa activity/metabolism affects the glycolytic pathway, resulting in a lactic acid build up in the surrounding medium. This build up of lactate is due to the saturation of mitochondrial lactate transporters which causes the cytoplasmic pH to drop (Jones and Connor, 2004). Reducing temperature helps prolong the life of spermatozoa by reducing metabolism as well as bacterial growth (Morrell, 2011). Transportation and preservation of semen must therefore be done at low temperatures to preserve the fertilizing potential of the spermatozoa. However, a decrease in percentage motility of spermatozoa can result from rapid changes in environmental temperature (Payan-Carreira, 2011). Spermatozoa have been observed to swim towards regions of warmer temperature (Bahat *et al.*, 2003). Since there is a temperature difference in the reproductive tract

(2^{o}C between the cooler tubal isthmus and the warmer tubal ampulla), this is one reason spermatozoa swim towards the oocytes.

2.1.3.9 Light: Exposure of spermatozoa to light reduces their lifespan. Spermatozoa should be protected against strong light Herman (1994). Otite (2000) noted a reduction in respiration rate of spermatozoa exposed to light, finally resulting in death of spermatozoa. Semen should thus be collected and stored in opaque bottles in order to preserve their lifespan. An ideal preservatory medium should be capable of reducing the activity of spermatozoa in order to maintain the stability of the medium.

2.1.3.10 pH: Canine spermatozoa is very sensitive to the ph of the extender. Carr *et al.* (1985) observed that motility increased when the pH of the medium was increased to 7. Decreasing pH led to greater inhibition of spermatozoal activity. Crusco dos Santos (1999) also observed a similar trend that a pH decrease of the extender promotes an integrity damage of the spermatozoal membrane, consequently interfering with the fertilizing capacity of the sperm.

One major factor in semen extension and preservation is maintaining the pH of the medium. Usually the pH of the medium drops with increased activity. The addition of carbon dioxide helps in maintaining pH as it makes spermatozoa immobile. Researchers recommend the use of the sperm rich portion in semen extension as the third fraction activates the sperm cells and thus increases their motility and hence the pH of the medium drops.

Wales and White (1958) proposed that canine spermatozoa can survive between a pH range of 5-10, with an optimal pH range of between 7 and 8.5. They also found that canine spermatozoa were more tolerant to alkalinity than acidity.

2.1.3.11 Time of collection: Although it is advised that semen be collected early in the mornings or late in the evenings (Otite and Egbunike, 2000), in order to reduce the environmental heat imposed on the dog in the tropics due to high temperature extremes, it is advised that semen be collected based on weather conditions rather than time of the day.

2.1.3.12 Libido: The willingness of the dog to breed is an indication of the quality of semen (Otite and Egbunike, 2001). As the sperm reserves increases, the desire to mate is increases.

Otite (2000) observed an increase in volume in dogs with high libido. However, the dogs reaction to the vagina would also be an important factor in determining the total semen output.

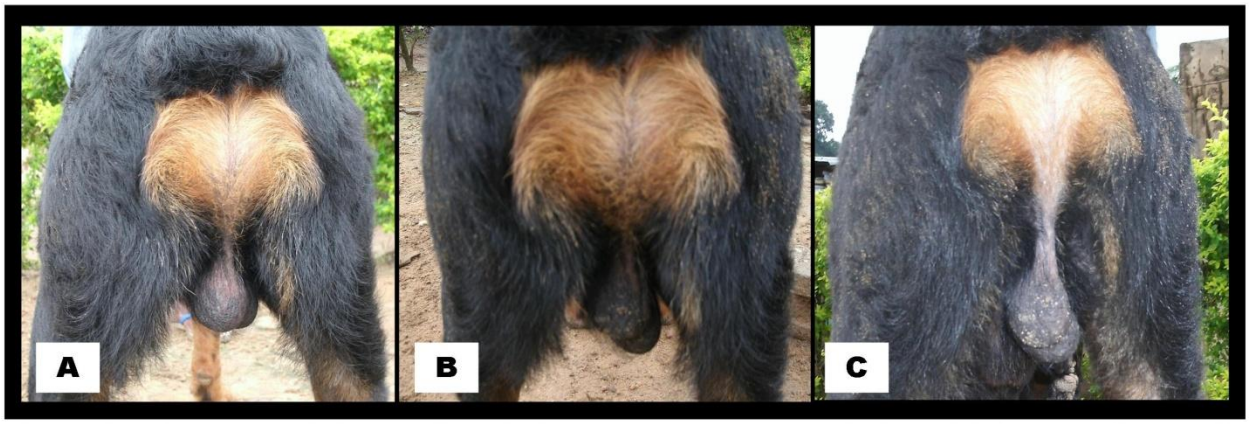


Fig. 2.4: Distends of the testis during temperature regulation. (A) Shows the testis drawn closer to the body during cool temperature. (B) shows the distend of the testis during normal environmental temperature. (C) The testis released farther away from the body during warmer temperature.

2.1.3.13 Starvation: Teitelbaum and Grant (1956) observed that starving dogs prior to collection increases the total volume of the ejaculate. Digestion diverts the direction of blood surge in the sense that nutrients that are carried by the blood at a time when we need the attention of blood flow to be directed to the penis.

2.1.3.14 Health: High body temperatures or fevers might adversely affect the dog's response to semen collection as well as reduces the quality of semen (Grognet, 2004). Since spermatogenesis only occurs when the testis temperature is below body temperature, this suggests that there is a maximum temperature the testis can function. A rise in body temperature would also raise the testis temperature. Even though this temperature might be below rectal body temperature (as the difference between both temperatures is about 3.6^{o}C (Saypol *et al*, 1981), this could still exceed the testicular critical temperature and in some cases might be irreversible. Healthy dogs should produce semen with not more than 10 000 bacteria per ml (Paclikova *et al.*, 2006). However, this figure might be altered in times of sickness. Just as a prostate gland infection could result in aspermia, damage to testicular tissue can result in poor sperm production or even aspermia as well as azoospermia (Grognet, 2004).

Dogs should therefore be examined prior to stud use. In cases where a clinical thermometer is absent, a dry nose is indicative of a fever and poor health conditions in dogs.

2.1.3.15 Unilateral Cryptorchidism: Although unilateral cryptorchids can produce litters, their sperm volume and concentration are reduced (Memon and Tibary (2001). In agriculturally advanced countries, the use of unilateral cryptorchids in breeding (irrespective of the method of fertilization) is prohibited as cryptorchidism is a recessive trait and thus heritable (Johnston *et al.*, 2001). Without examination, unilateral cryptorchidism in a male might be unsuspected as unilateral cryptorchids do show abnormal sexual behaviour due to their production of testosterone (Matheeuws and Comhaire, 1989) in addition to showing sexual desire, they are capable of having an erection (Badinand *et al.*, 1972).

2.1.4 Semen collection in the dog

All methods of semen collection must be aimed at collecting the semen in a manner which would reduce the amount of contamination as well as induce the least stress on the dog. Such a method of collection should be capable of producing optimum quantity and quality of semen. Dogs react to the collection process in different ways. It is thus advisable to get the dog accustomed to the method of collection prior to using him for semen collection. Various methods of initiating the dog to the collection process include bringing the dog in contact with the collection equipment, gradually massaging the dog at the bulbus glandis in a manner that would not over excite the dog (Otite and Egbunike, 2000). This can easily be done by speaking in a subtle manner to the dog while stroking the dog simultaneously.

Immobility of the animal to be mounted is an important factor in breeding. Usually, a male dog would willingly mount a standing female or even another male. A similar trend can also be observed in pigs. It is partly for this willingness to mount same sex in the absence of the opposite sex that majority of animal lovers claim that homosexuality in humans is an acquired trait rather than an inherited trait. Collecting semen can also be enhanced by providing an immobile female or male close to the stud at the time of collection. Equally, a gloved hand smeared with vaginal extracts from a female on oestrus would be enough to arouse the male to be used at stud. There are numerous methods of semen collection in the dog, each with its own advantages and disadvantages. Among these are:

Fig. 2.5: Tie during natural mating caused by the enlargement of the bulbus glandis of the male as well as the contraction of the constrictor vestibulae muscles of the female.

2.1.4.1 Massage Technique: This is the cheapest and easiest method of semen collection in the dog. It is therefore the most commonly used method (Kutzler, 2005). Digital manipulation is performed by massaging the dog around the bulbus glandis in order to stimulate an erection. Semen is then collected into a measuring cylinder with the aid of a funnel. The major disadvantage of this method is the semen obtained is usually contaminated. Just like milk, semen is easily attacked by numerous bacteria and hence exposing semen to the atmosphere increases the likelihood of contamination.

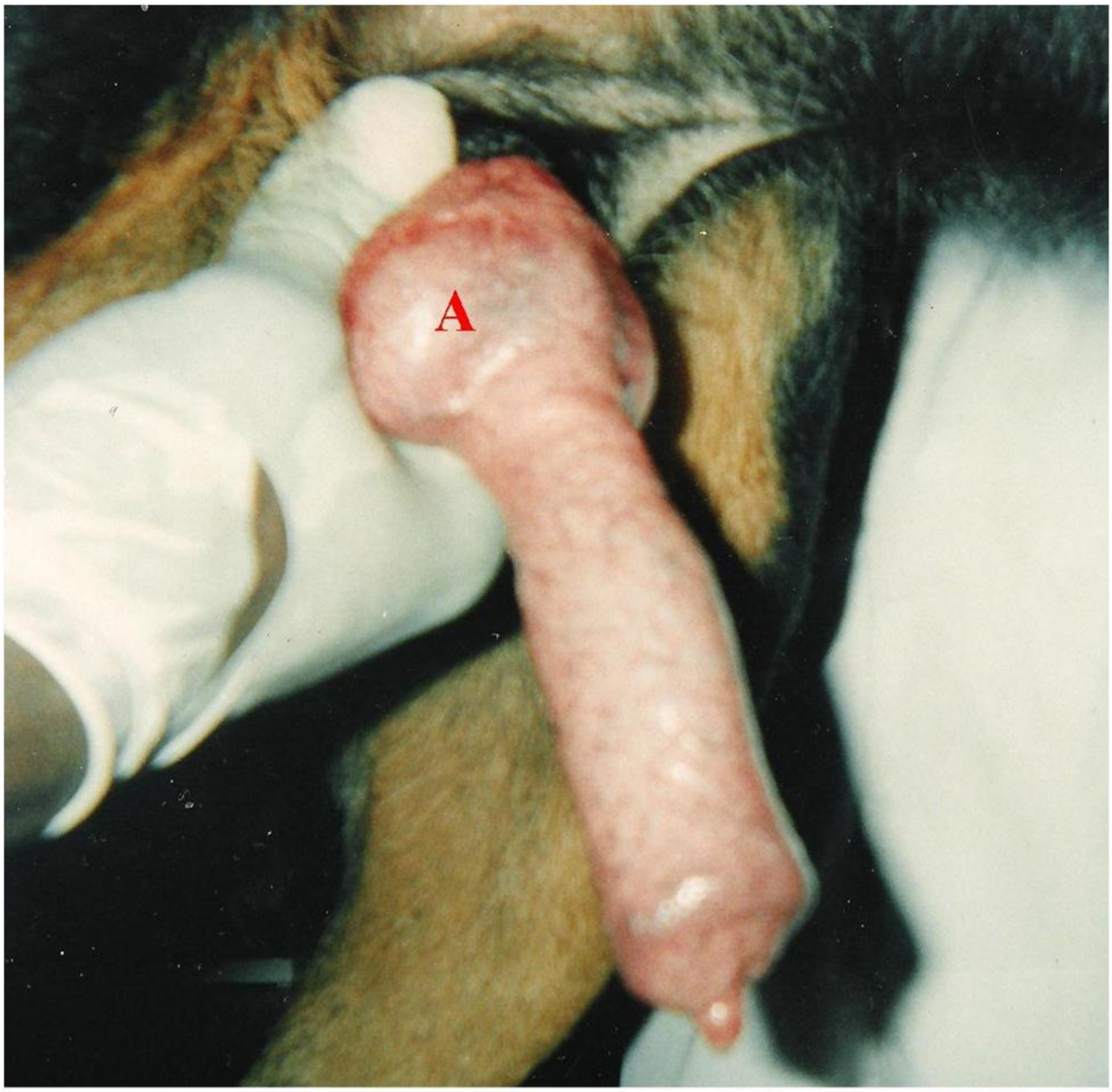


Fig. 2.6: The enlargement of the bulbus glandis (A) of the male dog during normal erection

2.1.4.2 Artificial Vagina: To date, the most common canine artificial vagina is the Harrop's Canine Artificial Vagina (Harrop, 1954). The typical canine artificial vagina is made up of an outer rubber casing with a lumen. A latex liner is fitted to this as in a typical bovine artificial vagina. Warm water is introduced between the linings and outer cover to give the desired vaginal temperature. A cylindrical bladder is fitted to the water space. This bladder is connected to a

pulsator. This method of using expensive and fanciful artificial vaginas is not necessary as other cheaper means exist today. Otite and Egbunike (2000) observed that the quality of semen collected under this method was not superior to that obtained using cheaper equipment such as the balloon artificial vagina.

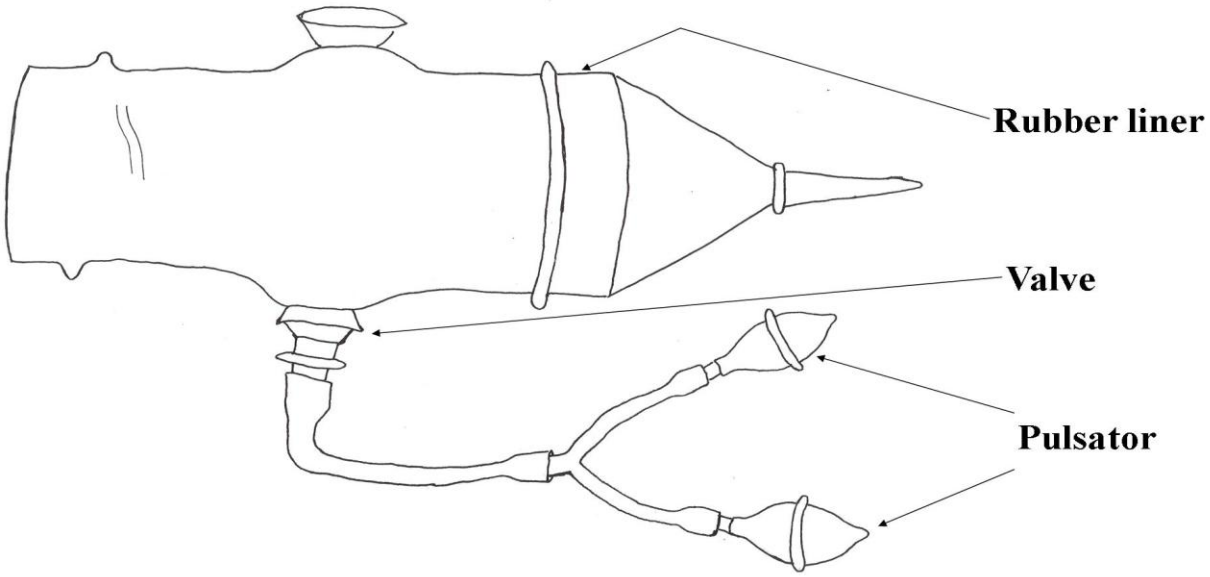


Fig. 2.7: The Harrop's Canine Artificial Vagina.

Fig. 2.8: The balloon artificial vagina

2.1.4.3 Electro ejaculator: This method employs the use of electric current to bring about an ejaculation. The electro ejaculator consists of an electric generating device, a power control and a probe (specific for the animal species in which semen is to be obtained). The probe is gently inserted into the rectum of the male by the handler. This is followed by varying the current from the current source by means of a rheostat. By this, the prostate and ampullae are stimulated causing the dog to release his ejaculate (Ohl *et al.*, 2007). This method is usually faulted in canine breeding as there is the risk of the animal going into muscle tetany or developing cramps. Also this method produces lots of distorted spermatozoa due to the forceful nature in which the spermatozoa are ejaculated. Harrop (1960) observed unsatisfactory results with this technique as the semen collected is usually contaminated with Urine. However, Ohl *et al.* (1994) did not observe any detrimental effects of this method of semen collection on the motility of spermatozoa.

2.1.4.4 Balloon Artificial Vagina: This method has been used with great success (Otite and Egbunike, 2000). The artificial vagina consists of a balloon attached to a collection bottle. Prior to collection, the penis of the dog is whipped with warm water to remove all dirty substances which would contaminate the ejaculate. The sheath surrounding the penis is then pulled backwards and the penis is inserted into the balloon which serves as a vagina. This method is relatively cheap to use as the balloon helps in insulating the dog thus using the heat produced by the penis on the dog. This eliminates the need for a heat source to pump in warm water as in the case of other artificial vaginas. The handler grasps the penis of the dog and massages the bulbus glandis (Fig. 2.6) at the same time producing a gentle pulse just as the bitch would do in natural mating. This is usually followed by an erection and then ejaculation. Unlike the massage technique, the artificial vagina is relatively more hygienic as the semen has little or no contact with the surrounding since it flows directly into the collection bottle. The collection bottle is also opaque thus enhancing the shelf life of the spermatozoa. Results obtained from the use of the Balloon Artificial Vagina shows that it can produce a high volume of ejaculate just like the other methods of semen collection.

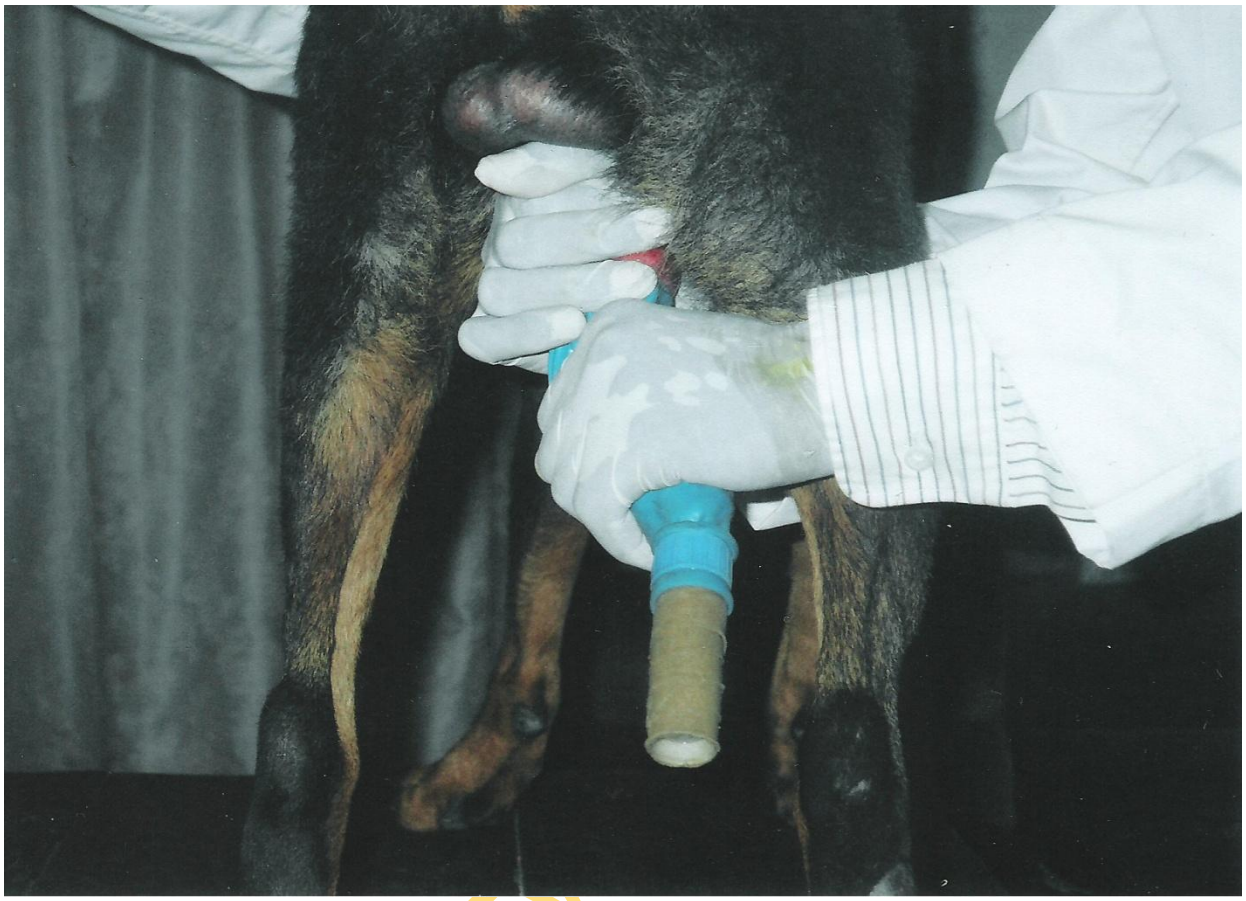


Fig. 2.9: Semen collection using the balloon artificial vagina.

2.1.5. Semen Preservation and extension

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

(i) It must be able to provide nutrients for the spermatozoa.

(ii) It must be capable of maintaining the osmotic balance of the medium.

(iii) It should enable multiple inseminations from a single ejaculate by increasing the volume of semen.

(iv) It must have high buffering ability to prevent change of pH.

(v) It should be capable of preventing bacterial growth in the medium.

(vi) It should be able to prevent death of spermatozoa due to direct contact between extender and spermatozoa or due to cold shock as in the case of freezing and cryopreservation.

(vii) It should not affect the visibility of spermatozoa during microscopic examination.

(viii) It must be able to maintain motility of spermatozoa within the recommended level.

The extenders in existence today are on the increase with new extenders doing either just as well or better than the older ones. The first extender was produce by Anderson in1945. This was a simple sugar or salt solution intended solely as a diluents to increase the volume of semen. Modern extenders include: Tris buffered extender (Battista *et al*., 1989; Olar *et al*., 1989; Thomas *et al*., 1993), Tns/Tes (England, 1992), coconut water extender (Cardoso *et al.*, 2002), Zobo extender (Otite and Egbunike, 2002), skim milk extender (Romagnoli, 2002).

2.1.5.1 Buffers: Buffers are required more for semen preservation rather than semen extension as the pH usually drops with time and hence if semen is to be used immediately after collection the use of buffers would have less significance in achieving fertility. Buffers are thus needed to maintain the pH of the medium which drops with time when semen is preserved. There are two typed of buffers. These are inorganic buffers and organic buffers.

2.1.5.1.1 Organic Buffers: The term organic here is entirely different from the term "organic" in food production as organic buffers simply refer to buffers of natural origin irrespective of whether they are obtained from organic farming or not.

2.1.5.1.2 Inorganic Buffers: These include phosphates, glucose, fructose, citric acid etc.

Phosphates: Phosphate buffers are widely used in semen extension. It has been observed that canine spermatozoa are able to maintain high motility in phosphate buffers (Wales and white, 1958). Phosphates are effective when it comes to storing semen above freezing point.

2.1.5.2 Citrates: Salisbury *et al.* (1941) observed that the addition of citrate to mammalian semen prolongs survival. Sodium citrate also aids in dispersing fat globules so that sperms cells can be seen more clearly under the microscope. Canine spermatozoa have been noted to thrive

better in extenders consisting of egg yolk citrate than semen preserved in egg yolk phosphate (Bartlett, 1962).

2.1.5.3 Glucose and Fructose: These have good buffering ability as they help maintain the osmotic balance of the medium. In addition to the buffering properties of glucose and fructose, their inclusion in semen extenders is beneficial as not only do they help maintain the pH of the medium but also nourish the spermatozoa by providing readily available energy for the spermatozoa.

2.1.5.4 Additives: Certain additives are required in order to maintain the viability of extended semen during preservation:

2.1.5.4.1 Antibiotics: Most commonly used are penicillin and streptomycin. Semen has a high biological value and just like other proteins with high biological value, semen is attacked by bacteria. Dede (1974) observed semen to be rich in bacteria flora. England and Lofstedt (2000), estimated the amount of bacteria in semen to exceed 10,000 per ml. Besides attacking individual spermatozoa, bacteria compete with spermatozoa for available nutrients thus resulting in the death of spermatozoa through direct attack as well as rapid pH drop. Yaniz *et al.* (2010) observed that the addition of the antibiotic gentamicin had significant antimicrobial activities thus improving semen quality. Andrabi (2007) also found a combination of antibiotics (gentamycin, tylosin and linco-spectin) to be useful in semen extension. His work using this combination was on bovines. No reliable data exists for this in canines.

2.1.5.4.2 Egg yolk: Egg yolk is rich in essential nutrients such as glucose, several amino acids, fats and oils, soluble vitamins and carotene, iron, riboflavin, phospholipids and folic acid (Earle and Smith, 1993). Egg yolk also contains antioxidants which are useful for cell membranes (Huopolathi *et al.*, 2007). Verstegen *et al.* (2005) observed that the addition of egg yolk had positive effects on semen.

Besides providing essential nutrients for spermatozoa, Farstad (2009) observed that the addition of egg yolk to the storage media reduced mortality arising from cold shock in spermatozoa preserved below freezing point. Egg yolk also prevents or restores the loss of phospholipids from the membrane during cold storage (Farstad, 2009). Although egg yolk proves to be very beneficial in semen extension, there exists the scare of the spread of zoonotic diseases such as

influenza through egg yolk. As a result of this, there is currently a search for an alternative to egg yolk.

2.1.5.4.3 Coconut Water: Coconut water (*Cocos nucifera*) has produced satisfactory results in canine semen preservation (Ross, 2005). Cardoso (2002) proposed that a combination of glycerol and coconut water can be used to cryopreserve canine spermatozoa. Coconut water is rich in 12 essential amino acids such as cystine, methonine, valine, and leucine. Coconut water is also a good source of vitamin B1 and B2. The pH of Coconut water is approximately that of blood (Campbell-Falck *et al.*, 2000). The ph of blood in dogs is the same as for dog semen (Mann, 1969). This explains why Ross (2005) and Cardoso (2002) obtained satisfactory using coconut water. Another attribute of coconut water is that it does not alter the colour of the semen sample making visibility possible.

2.1.5.4.4 Zobo (*Hibiscus sabdarifa*): Otite and Egbunike (2002) observed that zobo extenders could be used to maintain canine semen motility at room and refrigeration temperature. However, to date there exist no information on the suitability of zobo for cryopreservation. Zobo is rich in ascorbic acid and contains a high amount of energy. Zobo is also low in fats and its pigments are believed to play a role in the prevention of oxidative damage in living systems (Wang *et al.*, 2000) as they are a great source of natural antioxidants which protect the body from damage.

Table 2.1: The nutritional content of Zobo (*Hibiscus sabdarifa*)

Botanical Name	Common name	Local name	Food energy (Cal)	Moisture (%)	Protein (g)	Fat (g)	CHO (g)	Fibre (g)	Ash (g)	Ca (mg)	P (mg)	Fe (g)	Carotene (g)	Thiamine (g)	Riboflavin (mg)	Niacin (mg)	Ascorbic acid (mg
Hibiscus sabdarifa	Zobo, Sorrel, Osce ille de Cuinee	Zobo	276.00	16.60	4.90	0.30	74.00	15.80	4.50	55.00	163.00	163.00	4,135.00	0.14	0.45	1.20	54.00

Source: FAO, 1968

Note: Values given per 100g

2.1.5.4.5 Skim milk: Skim milk based extenders are currently one of the best to date. Skim milk is useful as it contains fat globules and lipoprotein which provide additional protection to spermatozoa during cooling thus aiding in reducing the effects of cold shock arising from the freezing of spermatozoa (Kiso *et al.*, 2011). Rota *et al.* (2001) claim the results obtained from using skim milk to be at par with those obtained from tris based extenders. However, Crusco Dos Santos *et al.* (1999) observed tris-fructose-citric acid to be superior to skim milk extender. Due to the global fear of the spread of diseases such as avian influenza, there has been some restriction over transportation of egg based cryopreserved extenders. Abe *et al.* (2008) have used a combination of skim milk/glucose and glycerol as a potential replacement for egg yolk. Skim milk must however be heated to destroy its spermicidal properties (Flipse *et al.*, 1954).

2.1.5.4.6 Orvus ES Paste (OEP): The addition of OES by Tsutsui T (2000) has shown that OES helps maintain acrosome integrity as well as post thaw motility of canine semen. However, there exists little information on OES in canines.

2.1.6 Cryopreservation

Cryopreservation entails the preservation of semen at sub zero temperatures. This is achieved by immersing samples in compressed (liquid) gases such as nitrogen or carbon dioxide. Liquid Nitrogen which boils at -196^{o}C is the most commonly used for cryopreservation.

This is partly because unlike other gases such as hydrogen, nitrogen is not combustible and makes up for as much as 78% of the atmosphere. The major hazards of Liquid Nitrogen is its ability to displace oxygen leading to asphyxiation. This can easily be overcome by installing oxygen monitors in areas where liquid nitrogen is stored. Other gases such as carbon dioxide have been used to preserve canine semen (Noakes *et al.*, 2001).

Although seminal plasma has pH buffering ability, it contains inhibitors of immune responses including protective components that coat sperm (Suarez and Oliphant, 1982; Dostal *et al.*, 1997). However, during cryopreservation, only the sperm rich portion is used. This would facilitate the reducing of the effects of cold shock as well as aid in the removal of water from the spermatozoa, thus reducing the buildup of ice crystals within the spermatozoa.

Table 2.2: Boiling Point (BP) of various cryogenic gases

Gas	NITROGEN	OXYGEN	ARGON	HYDROGEN	HELIUM
BP, °F	-320	-297	-303	-423	-452
BP, °C	-196	-183	-186	-253	-268
Volume Expansion	696	860	696	850	745

Source: ASA, 2006

2.1.7 Fertilization and implantation in the dog

Unlike spermatozoa which can stay motile in the female tract for as long as 11days (Doak *et al.*, 1967), the oocytes have a much shorter lifespan ranging from 12 to 48 hours (Lofstedt, 2004; Tsuitsui, 1989) after ovulation. Spermatozoa are brought to the oocytes for fertilization through contraction of the uterine walls. This process is usually fast and can range from seconds to a few minutes. The motility of the spermatozoa comes into play in the final process of getting in contact with the eggs. Upon contact with the oocytes, the acrosome (head cap) attaches to and dissolves the zona pellucida (wall surrounding the oocyte) in order to allow contents from the nucleus flow into the oocyte. This process occurs in the uterine tube. The fertilized embryo then begins its travel down the uterine tube to the uterus. This process in dogs last about the longest in all farm animals hence the embryos arrive at the uterus about 10 days after the LH surge, 7 days longer than in the cow. Intra uterine migration occurs once the embryos arrive into the uterus. This is one method of ensuring an even distribution of embryos between the uterine horns. Implantation is however established about 17-18 days after the LH surge (Lofstedt, 2004). This could however be delayed till 22-23 days (Thatcher *et al.*, 1994).

Unlike many other farm animals, the size of a litter cannot be estimated from the number of follicles as a canine follicle can contain more than one oocyte making it possible for an entire litter of pups to be born from a single follicle. The total number of oocytes from one follicle can be as much as 17 (Loftsedt, 2004) to 20 (Reynaud *et al.*, 2010). Dogs are thus said to possess poly-ovular oocytes. Litter size in dogs is highly dependent on breed size (Borge *et al.*, 2011; Thomassen *et al.*, 2006) with the larger breeds having a higher litter size than the smaller breeds except the Pekingese which has a fairly large litter size (Lofstedt, 2004).

Fertility or pregnancy rate in the bitch is at its peak (above 85%) during the third and fourth oestrous period and decreases thereafter. Thomassen *et al.* (2006), observed better conception in bitches less than 6years of age. In many polytocous animals, there exist the possibility of superfecundation hence a bitch bred with more than one male, either artificially or naturally can produce offspring from more than one sire. It is for this reason that in fertility studies, researchers always ensure that the bitch does not come in contact with other males during oestrous as there is the likelihood of the bitch accepting multiple males and hence producing unwanted puppies.

2.1.8 Artificial insemination in dogs

Although Leeuwenhoek (1678) and Hamm, were the first persons to see sperm, the first recorded Artificial Insemination was performed in the dog by Spallanzani (1784). Although the awareness of artificial insemination is still low in Nigeria, its use in other regions of the world is on the increase.

There are numerous advantages of artificial insemination in the canine industry (Herman *et al*., 1994). Among which are:

(i) Solution to complicated or impossible natural mating: certain conditions might prohibit or complicate natural mating. Artificial insemination is a way of inducing fertilization in such instances. Such situations which might inhibit natural mating are anatomical defects such as weak spine or limbs or in some cases narrow or tight vulva or vagina. Natural mating may also not be possible due to size disparity between the stud and the bitch.

(ii) Eliminates the stress caused due to behavioural problems as in the case of premature erection i.e. when a male gets erect before penetrating the vulva. This makes the desired tie that occurs during mating impossible.

(iii) Reduces stress of transporting breeding animals: maintaining a pure line is very important in the canine industry. One major advantage of maintaining a pure line is to be able to predict the outcome and performance of dogs. An example of this is height or weight at a certain age. Due to this importance of maintaining purity, locating distant strains may be arduous. In certain cases, males may be located far away which would increase the cost of transportation. The use of artificial insemination eliminates the cost of transporting breeding animals across long distances or international borders.

(iv) Improves breed standard: The use of progeny tested, top champion lines in artificial insemination enhances the genetic potential of dogs. Breeders no longer have to settle for what is available within their vicinity but rather they are able to breed using top quality studs located worldwide and thus have access to a wider gene pool.

(v) Reduces stud evaluation time: Artificial insemination reduces the time frame needed to evaluate studs due to the fact that a high number of offspring can be obtained using artificial insemination as opposed to the production of a few puppies per year. As a

result, a large number of offspring can be evaluated for desirable or undesirable traits passed on from stud to offspring.

(vi) Reduces cost of maintaining studs: the need to maintain studs by breeders can be either reduced or eliminated as breeders now have access to frozen semen or can request shipment of semen from other breeders.

(vii) Eliminates danger of fight outbreak: Not all bitches allow mounting while on oestrus and would react by attacking the stud or handler. This usually results in unwanted fights and injuries. With artificial insemination, both stud and bitch do not have to meet physically. Thus eliminating the danger of injury.

(viii) Controls Disease: Artificial insemination is one way of reducing the effects of disease transmission in animals. These include both sexually and none sexually transmitted diseases such as brucellosis, leptospirosis, canine distemper and canine parvo virus. Also as a result of both stud and bitch not being in contact, the spread of disease causing pests such as fleas and ticks is eliminated. In addition, the use of semen is now highly regulated by many bodies.

(ix) Increases stud life/usage: Artificial insemination increases the rate of use of a dog at stud by many folds. With artificial insemination a dog could sire over a hundred puppies per year and is thus a new source of income for stud dog owners.

(x) Provides better records: Artificial insemination aids proper record keeping in the canine industry. Breeding can be regulated as opposed to free range or natural breeding. This helps prevent the reproduction of unwanted inherited traits. Distant related animals can easily be identified among breeding animals and is thus a way of maintaining vigour among canine breeding units.

(xi) Increases choice of stud: With the use of the internet, a breeder can access the internet and source for a dog that meets his desires independent of country of origin and have the semen shipped to him/her.

2.1.9 Techniques of Artificial Insemination

Artificial Insemination can be performed either surgically or non-surgically. Each has its own advantages and disadvantages.

2.1.9.1 Non surgical insemination: The most common form of non surgical insemination is intra vaginal insemination. Intra vaginal insemination is widely used and also produces a high conception rate when inseminating fresh or chilled semen. However, it is widely considered the least effective in terms of conception rates using frozen semen. Linde-Forsberg (2001) and Nizański, (2006) observed that this method produced less litter sizes using fresh, chilled or frozen semen than when semen was deposited directly into the uterus. Silva *et al.* (1996) observed no difference in conception rate between intra vaginal insemination and the intra uterine method. Intra vaginal insemination is relatively easy to perform as it involves simply passing a sterilized catheter through the vagina of the bitch up to the opening of the cervix and depositing the semen at the beginning of the vaginal tract. The bitch is then raised in an inclined position (Fig. 2.11) and a gloved finger or sterilized rubber pipe is placed slightly into the vagina to stimulate vaginal and uterine contractions. This muscular contractions enhance the passage of spermatozoa through the uterine cavity (Suarez and Pacey, 2006).

2.1.9.2 Trans-cervical insemination: Trans-cervical insemination is very effective when inseminating thawed frozen semen or when males have poor semen quality such as low sperm count. It involves the use of an endoscope equipped with a 70 degree telescope and fibre optic light source to visualize the cervical entrance. This method can also be used to visualize the uterus and the uterine horns. Thomassen *et al.* (2006) observed that this method optimized conception in bitches irrespective of the number of times bred. In trans-cervical insemination, a catheter is inserted through the cervix in order to deposit the semen directly into the uterine horn, where fertilization takes place. This method the deposits semen as close as possible to the oviducts. It is simple as it bypasses the need for anaesthesia or sedation.

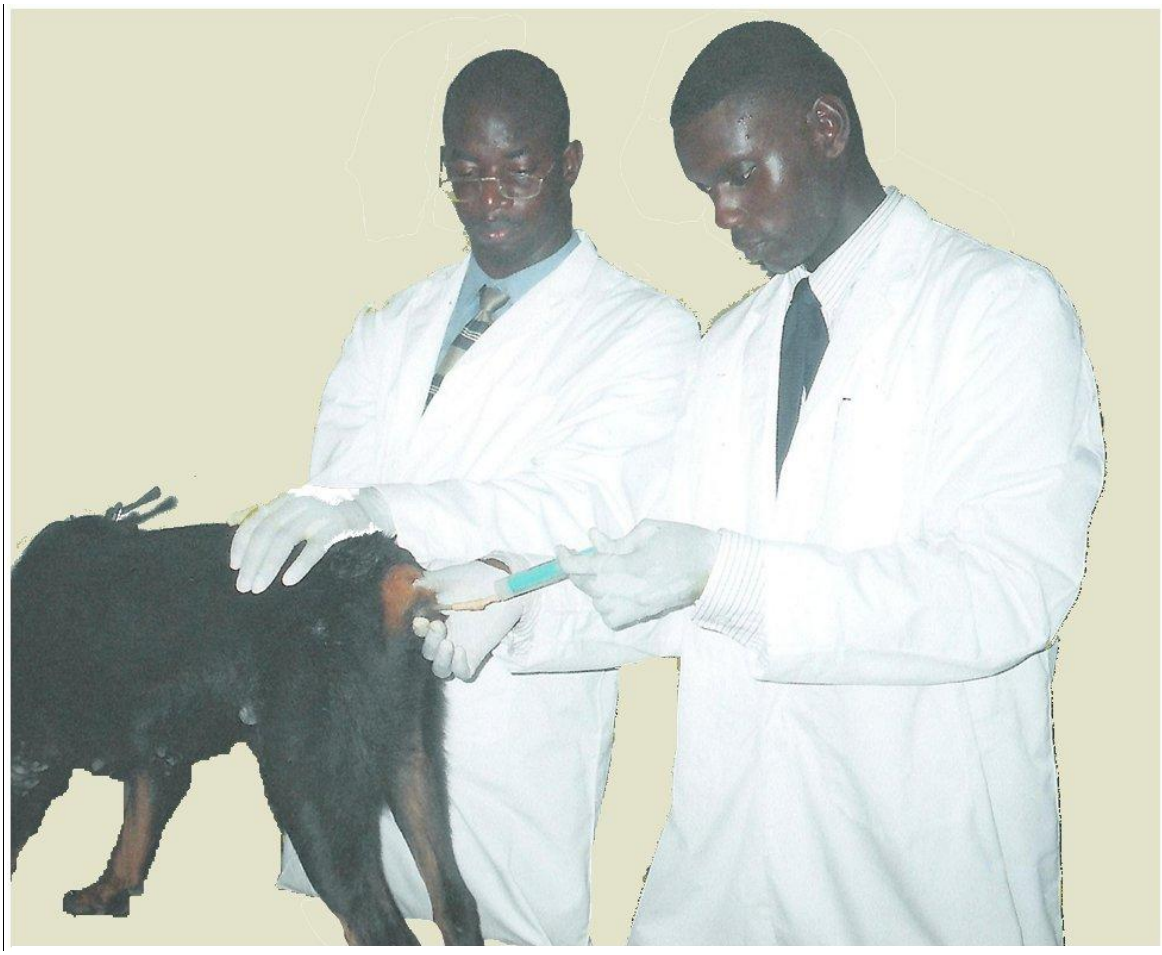


Fig 2.10: Intra vaginal insemination performed on a Rottweiler

The use of the endoscope enables the insemination process to be viewed or video thus confirming the location of semen deposition and the effectiveness of the process. Trans-cervical insemination can also be done using a Norwegian catheter.

2.1.9.3 Trans-cervical insemination with the Norwegian Catheter: This is a simpler method of trans-cervical insemination and requires less specialized hands. The results when mastered are as effective as those obtained from the endoscope method. Success rates as high as 84.5% and 71% have been reported (Linde-Forsberg *et al.*, 1999), (Thomassen *et al.*, 2001) respectively. The Norwegian Catheter consists of a plastic guide (sheath) with a hollow passage, and a stainless steel catheter.

During Insemination, the sheath is inserted into the vagina and a hand used to feel the abdomen of the bitch in order to locate the cervix. This he does by also using the sheath to direct him

along the vaginal tract. The inseminator then uses his hands to align the rigid sheath with the cervix in order to locate the entrance of the cervix. He then passes the stainless steel catheter through the sheath and into the uterus via the uterine opening and then deposits the semen into the uterus.

One advantage of this method over all others (excluding the surgical method) is the ability to feel the texture of the cervix which dilates at the optimum time of insemination.

Fig. 2.11: Elevating the hind legs of a bitch after Artificial insemination

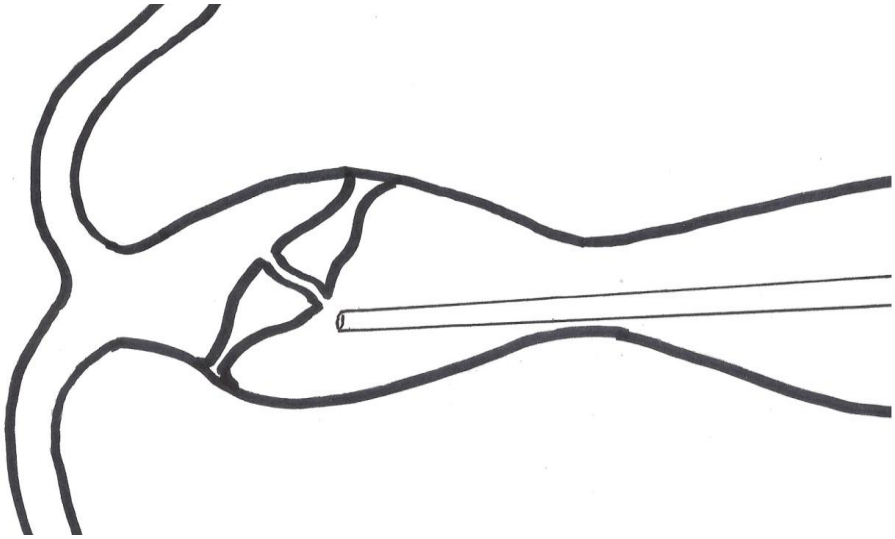


Fig. 2.12: The angular positioning of the cervix which makes trans-cervical insemination in bitches possible only through manipulation of the cervix

2.1.9.4 Surgical insemination: In surgical insemination, an incision is made in the abdominal region of the bitch in order to locate the uterus. Semen is then injected into the uterus unlike the trans-cervical method which only allows location of the cervical opening. This method is useful in that it gives an opportunity to examine the uterus of the bitch especially in the case of suspected ovarian or uterine disease. This method is the most effective as corrections to the uterus can be made prior to insemination in order to enable conception to occur.

In practice, this method is not important when using fresh or chilled semen as intra vaginal insemination which is relatively easier, produces a satisfactory conception rate. Initially, its use in frozen semen insemination was thought to be of invaluable importance but this has been proven wrong as recent studies have shown that it is not significantly superior to the transcervical insemination technique in terms of conception rate (Fukushima *et al*., 2010). Both methods are thus effective when using males with low sperm cell-count. The major problem associated with this method is its cumbersomeness as well as the use of anaesthesia which imposes stress on the bitch. Since this is a surgical process, many countries frown at this method of insemination (England & Millar, 2008)

2.2.9.5 Intratubal Insemination: Another form of semen deposition in artificial insemination is intratubal insemination. However, little information exists on the effectiveness of depositing semen this far in the reproductive tract of the bitch. Kim *et al.* (2007) observed a pregnancy rate sof Kim *et al.* (2007) did not take into consideration the best insemination site of the uterine tube nor did it estimate the minimum spermatozoa to be inseminated. The results might imply that there is no significant advantage of this cumbersome method of artificial insemination.

2.1.10 Future concerns of artificial insemination using thawed frozen semen

Majority of the concerns of artificial insemination using thawed frozen semen have been addressed over time. These include:

(i) **Conception Rate:** Initially poor results were obtained from the use of frozen semen as spermatozoa live for a maximum of 12hrs in the vaginal tract. Improper timing as well as method of insemination may have been a major reason for the low results obtained on the field using frozen semen. This has been overcome by proper knowledge of the physiology of the bitch which is distinct from all other mammals. It

is thus possible to determine the exact period the bitch ovulates as well as the optimum time for insemination.

Another reason for low conception rate was the method of insemination employed at the advent of the use of frozen semen. Artificial insemination at this time was performed using the intra vaginal technique which is ineffective (Tsutsui *et al.*, 2000). Tsutsui *et al.* (2000) and Thomassen *et al.* (2006) observed that the use of trans-cervical insemination as well as surgical insemination to bypass the vaginal tract and deposit semen directly into the uterus or uterine horn has been able to break this barrier.

(ii) **The safety of the insemination process on the health of the bitch:** Initially concerns were raised over the safety of the surgical insemination technique. However, this has been resolved through the advent of the transcervical technique. Certain countries now ban the use of the surgical method in canine artificial insemination (Linde-Forsberg, 2005)

(iii) **Sex Ratio:** Although this is not yet fully proven, many researchers believe that insemination time has no effect on the sex ratio of offspring (Roelofs *et al.*, 2006). Although the above might indicate so, there is still concern over this issue based on the knowledge of the disparity in swimming speed between male and female spermatozoa, and knowledge of the hormones associated with reproduction as well as the issue of inseminating at the optimum.

2.1.11 Optimum time for insemination

Numerous studies have been done on determining the stage of oestrus in the bitch as animals might show oestrous behaviour even when not in oestrus as in the case of nymphomania. There are several methods of determining the fertility period in the bitch. Many of these methods have been proven to be inadequate over time. Amongst these methods are:

(i) **Cytology:** Determining the time of ovulation by cytology is inefficient in bitches. The haemorrhagic discharge in the bitch is as a result of oestrogens. This discharge is erratic as it varies between bitches. However, since the bitch releases her eggs into a progesterone environment rather than an oestrogen environment, cytology is not an absolute way of determining the optimum time for insemination in the bitch.

Although opening of the cervix has been correlated with oestrus in dogs (Silva *et al.*, 1995; Vestergen *et al.*, 2001; Chatdarong *et al.*, 2002), observing other physiological changes in bitches on oestrus such as vulva dilation as well as blood colour which also occur under the effects of estrogens are not absolute ways of determining the best time for breeding. In addition, some bitches experience a condition known as "silent heat" in which visible exhibition of the signs of oestrus are absent. In addition, cell observation (red blood cells, parabasal cells, intermediate cells and superficial cells) could lead to erroneous conclusions.

(ii) **Day Count:** Counting the days from the onset of proestrus as done in the past does not usually yield desirable result in all instances and is the least assured method of ensuring that breeding is done at the optimum time. Dogs should be bred while on oestrous which in most cases begins after the 9^{th} day of proestrus. However, this only reflects an average as in some bitches, proestrus could last as short as 3 days, while in others it could last as long as 17 days (Threlfall, 2000).

(iii) **Male check:** Allowing the male to determine the right time of insemination in real terms is actually the actual day of oestrus (standing heat otherwise referred to as day one of the oestrus cycle). Some bitches will not allow mounting even when on oestrus and hence might be assumed as not being on standing heat by the breeder. Also, nymphomania in the bitch is not a rare occurrence (Evans and White, 2002). Many domestic bitches show signs of flagging (side moving of the tail) to other dogs and even their owners. Keeping a male on site constantly also results in more costs in the breeding unit.

(iv) **Electrical resistance test:** There are currently some devices for determining ovulation in bitches by means of measuring the electrical resistance of the vaginal mucus which undergoes characteristic changes during oestrus. An example of such is the Draminski ovulation detector. Such readings are inconclusive as the changes occurring as a result of the vaginal mucus are also as a result of estrogens.

All the above methods have been proven to be inadequate with time. There is thus a more efficient solution to the above problems. This includes the hormonal assay method.

2.1.11.1 Hormonal assay: This is by far the most effective method of determining the optimum time for insemination.

LH and FSH cause the formation of estrogens in the follicle. Estrogens increase the amount of LH due to its positive influence on the hypothalamus causing GNRH release. It is this increase in LH i.e. the LH peak that triggers the follicles to ovulate and discharge their oocytes usually occurring about 48hrs after the surge. The forming corpus luteum produces progesterone (note that initial basal progesterone is of adrenal origin). The overall rise in progesterone to 5ng/ml is an indication of ovulation in the bitch. Hence, only through hormonal assay are we able to know precisely the bitch's time of ovulation. Although determining the time of the LH source or identifying the point at which progesterone begins to rise till it gets to 5ng/ml are both effective (Root Kustritz, 2001), the later is preferred for the following reason:

(i) The LH surge lasts 24hrs which is relatively short and hence requires daily examinations which is costly.

(ii) Though it is assumed that ovulation occurs about 48hrs after the LH surge (average 24-48hrs).

(iii) Progesterone is formed by the corpus luteum which is an indication that ovulation has occurred.

Since the dog is unique among farm animals in that it ovulates premature oocytes which must mature before fertilization can occur, (usually 48-72hrs post ovulation (Concannon, 2004; Tsutsui *et al*., 2009), breeders are now able to ensure maximum fertility from a single mating, using either fresh, chilled, or frozen semen. A minimum of 150 to 200 x 10^6 spermatozoa is required for an effective insemination (Linde-Forsberg, 1991). The ova are viable for about 3 days after which they begin to deteriorate. Though a single mating 2 days after ovulation is adequate to produce a large litter size, it is advisable to have two mating (natural or artificial) done on the 4^{th} and 6th day after the LH surge Lofstedt (2004).

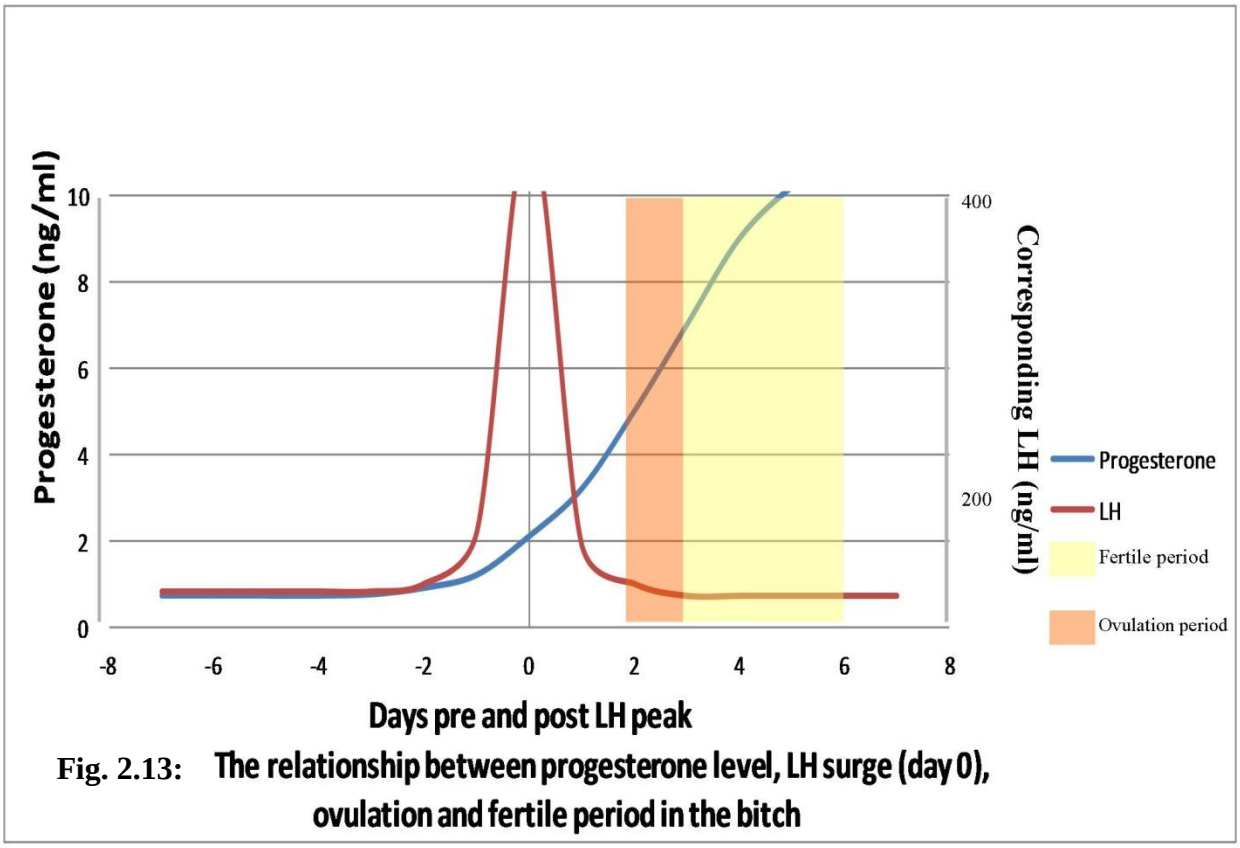


Fig. 2.13: The relationship between progesterone level, LH surge (day 0), ovulation and fertile period in the bitch

The volume of semen used is however necessary for several reasons among which are:

(i) Barrier from the cervical mucus: The cervical mucus produces a barrier to sperm preventing them from swimming pass this mucus. This effect is most felt at the border of the cervix due to the anatomy of the cervix which is more compact (Yudin *et al*., 1989). However, this is mostly effective against abnormal spermatozoa. This is more or less a means of sperm selection (Hanson and Overstreet, 1981; Barros *et al.*, 1984; Katz *et al.*, 1990, 1997).

(ii) Spermicidal nature of the vagina: Since the vagina meets the exterior of the bitch, it is open to different forms of infections. One way of preventing an influx of disease causing pathogens is through its acidic nature and immunological responses. Unfortunately this damages spermatozoa (Suarez and Pacey, 2005).

(iii) Negative effects of rapid transportation: When sperm are transported to the fallopian tubes especially those with high velocity (those that reach the cranial ampulla shortly after mating), majority of them are immotile or damaged. Overstreet and Cooper

(1978) after recovering spermatozoa in the cranial ampulla of rabbits observed that many were damaged and associated this result to the rapid wave of contractions associated with insemination. This rapid contractions result in overshooting of spermatozoa.

It is for these reasons that spermatozoa are released in high numbers by males. This overcomes the uncertainty of non fertilization.

CHAPTER THREE

EXPERIMENT 1

PRELIMINARY STUDY OF THE EFFECTS OF EXTENDERS ON SEMEN QUALITY

INTRODUCTION

Although Dog breeding is fast gaining awareness in Nigeria, access to distant strains for breeding purposes still remains difficult. There is awareness amongst dog breeders that in order to maintain or improve hybrid vigour, breeding is best done when distant related strains are used. However, locating and using distant related dogs can be highly cost implicative or fruitless. This is due to the high cost of transportation as well as poor maintenance of studs. There is also little knowledge of current trends in canine breeding techniques. As a result, dog breeding is still done using old techniques which yield poor results. Identifying the optimum time for breeding bitches is still done by day count or by cytology which are not absolute methods of determining ovulation in that some bitches are actually ready for breeding as early as the 5^{th} day from proestrus (Levy and Fontbonnes, 2007).

Though the above shows that breeding using distant related dogs in numerous instances would entail travelling with the bitch across long distances, this problem could have been solved if proper methods of animal breeding are utilized. Such contemporary methods include artificial insemination using preserved dog semen.

There is still a search for the ideal extender for use in canine semen extension and preservation. This chapter looks into the ability of different extenders to maintain semen quality when preserved at different temperatures.

3.1 MATERIALS AND METHODS

3.2 Experimental dogs

The experiment was carried out in Ibadan Oyo State Nigeria. Six studs were used for this experiment. The dogs comprised of 2 known breeds namely: the German Shepherd Dog (GSD) and the Rottweiler. These studs weighed between 38 and 40kg and where between two and three years of age. All dogs were in good health and had received all the required vaccinations. The experimental dogs were chosen based on body weight, as the weight of the dog plays a huge role in semen quality (Otite and Egbunike, 2001). A total of 3 ejaculates were obtained from each dog. These were then divided into portions based on the concentration of the spermatozoa in the ejaculate. These portions were extended in 4 different extenders in a completely randomized design.

3.3 Collection of semen

Semen samples were collected using the artificial balloon vagina (Otite and Egbunike, 2000). This consists of a sterilized balloon (which serves as an artificial vagina) attached to a sterilized opaque bottle serving as a collection bottle.

Prior to collection, the sheath of the dog was wiped with sterile water and then pulled backwards, exposing the penis. The penis was then inserted into the balloon. Ejaculation was achieved by gently massaging the bulb of the penis (bulbus glandis), thus initiating an erection (as the entire penis becomes engorged with blood) followed by ejaculation.

All collections were scheduled based on weather predictions as opposed to the time of the day in order to ensure that the dogs were not unduly stressed. This was done in order to ensure that semen was obtained without imposing stress on the dogs.

3.4 Semen evaluation

The following parameters were used in evaluating the semen quality of the dogs:

3.4.1 Volume: The ejaculate volume was obtained by carefully pouring the collected semen into a sterilized graduated measuring cylinder. Measurements were then obtained from the lowest level of the meniscus.

Fig. 3.0: different breeds of dogs used for this experiment. Above: Rottweiler. Below: German Shepherd Dog (Alsatian)

3.4.2 Concentration: The concentration of Spermatozoa was determined by the use of a Neubeur Haemocytometer (Egbunike *et al.*, 1976). Fresh canine semen was drawn up to the 0.5 mark of a red cell pipette. 0.25 percent formol-saline was then drawn up to the 101mark of the pipette. After shaking the mixture slightly, 3-4 drops were emptied away and then a drop of the diluted semen was allowed to run into the spaces between the cover slip and slide on either side of the ditch of a Neubauer haemocytometer. The total number of spermatozoa in 16 squares was counted and the sperm concentration calculated as follows:

No of sperm x 10 x 200 x 1000

10 representing the depth chamber of the haemocytometer while 200 represents the dilution rate.

3.4.3 Mass activity: The mass activity was done by placing a drop of semen on a warm slide and estimation was done under low power magnification (x10) of the microscope (Feyrer-Hosken, 1996). Classification was done using the following scores:

30% Low wave motion (poor)

40-70% Moderate wave motion (fairly good)

≥ 80% Vigorous wave motion (very good)

3.4.4 Percentage progressive motility: This was done using Walton's method of estimating motility (Walton, 1933). A drop of semen was placed on a warm slide and placed under a cover slip. Estimation of motility was then done by microscopic examination. In order to increase accuracy, 5 spermatozoa were counted from different fields of the slide and awards were given as follows:

5 - All spermatozoa present are motile.

4 - 4/5 spermatozoa present are motile.

3 - 3/5 spermatozoa present are motile.

2 - 2/5 spermatozoa present are motile.

1 - 1/5 spermatozoa present are motile.

0 - only oscillatory movements.

N- all spermatozoa present are dead.

The percentage motility was calculated by multiplying the result by 100 e.g. 4/5 x100 = 80%. Hence from the scores giving above, 5 means 100% progressive motility, 4 means 80% progressive motility etc.

3.4.5 Morphology: The number of spermatozoa showing morphologically atypical features were recorded as well as the percentage abnormal spermatozoa. Spermatozoa were classified abnormal by taking the following into consideration: Abnormal head (i.e. macrocephaly, microcephaly and bicephaly), coiled flagellum, bent midpiece with cytoplasmic droplet, bent midpiece without cytoplasmic droplet, bent flagellum with cytoplasmic droplet, bent flagellum without cytoplasmic droplet, proximal cytoplasmic droplet and distal cytoplasmic droplet (Howard 1993). Eosin-nigrosin stain (Blom, 1950) was also used to distinguish between live and dead spermatozoa.

3.4.6 pH: This was done by matching the colour produced by dipping a pH paper into a drop of the semen sample and matching this colour on the set of colours on the container of the pH paper (Nkanga, 1989).

3.5 Semen Extenders

Portions of the collected semen samples were preserved using 4 different extenders (see appendix for the composition of extenders). These were:

(i) Sodium-Citrate-Glycine Extender (SCGE).
(ii) Coconut Water Extender (CWE).
(iii) Skim milk Extender (SME).
(iv) Zobo Extender (ZE).

3.6 Semen Extension

The collected semen was warmed alongside the prepared extender to body temperature (37 ^{o}C) and both were mixed together by gently inverting the mixture. Extension was done at a rate of 100 million spermatozoa to 1ml of extender.

3.7 Semen preservation

2 different preservation temperatures were used to preserve the extended semen. These were: body room temperature (28^{o}C) and Refrigeration (4^{o}C).

3.8 Addition of Penicillin and Streptomycin

Penicillin and streptomycin were added to all the extenders at a dosage of 10^5 i.u./1000ml and 100.00mg/100ml respectively. (Otite, 2000).

3.9 Statistical analysis

The results obtained where Analyzed for statistical differences using the analysis of variance ($p<0.05$) and their means were further subjected to the Duncan's Multiple Range Test where applicable using SAS (2008).

RESULTS

At room temperature, Zobo Extender (ZE) had the highest progressive motility of 90.31% (Table 3.1). These results were however not significantly different from those of Coconut Water Extender (CWE), Skim Milk Extender (SME) but differed significantly from Sodium Citrate Glycine Extender (SCGE). All extenders had their initial progressive motility values above the recommended minimum of 60% (Lofstedt, 2004) for good quality semen. The Mass activity was however significantly highest in SME (77.50%). Results recorded showed that all three extenders differed significantly in their initial mass activity with SCGE having the least values or 73.50% (Table 3.2). SCGE showed the fastest drop in mass activity.

At refrigeration temperature, ZE had the highest initial progressive motility of 91.88% (Table 3.3). These results differed significantly from all other results with SCGE having the least progressive motility. All results however were above the recommended minimum value for progressive motility of top quality semen. SCGE showed the fastest drop in progressive motility at 4^{o}C. The mass activity of spermatozoa was highest in SME at 4^{o}C. These results did not differ significantly from those obtained from ZE (75%) but differed significantly from those of SCGE and CWE. Although CWE had the lowest mass activity at this temperature (67.92%), it did not differ significantly from SCGE.

ZE had the longest survival time of 27.83 hrs at 28^{o}C (Table 3.5). All results obtained with regards to the survival time at 28^{o}C were significantly different. SCGE had the lowest survival time of 5.83 hrs. The highest record of survival time of spermatozoa at 4^{o}C was recorded in CWE (18.78days). These results were however not significantly different from the results obtained from ZE but differed significantly from SCGE and SME. ZE had the highest percentage abnormalities at both storage temperatures. Nevertheless, these values did not differ significantly among the four different extenders (Table 3.5).

SEMEN PRESERVATION AT ROOM TEMPERATURE

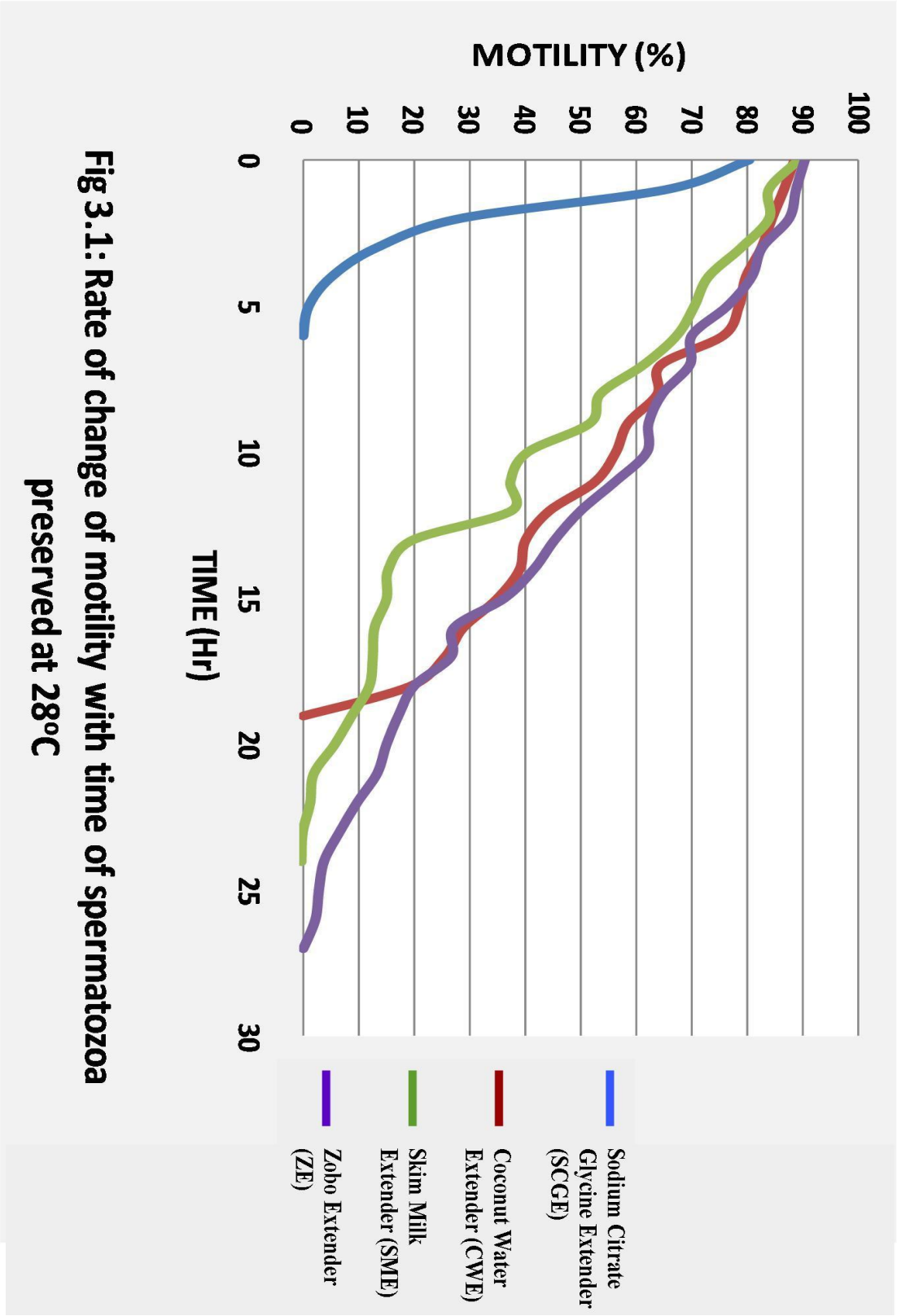


Fig 3.1: Rate of change of motility with time of spermatozoa preserved at 28°C

Table 3.1: Progressive motility (%) of canine semen stored at room temperature (28°C)

TIME (Hr)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
0	80.31[b]	88.44[a]	89.38 [a]	90.31[a]	0.26
1	65.63[b]	86.56 [a]	84.06 [a]	88.75 [a]	0.63
2	28.13[c]	84.38[b]	84.06[b]	87.50[a]	0.21
3	13.13[c]	82.50[a]	79.06[b]	82.63[a]	0.12
4	5.00[d]	79.69[b]	73.13[c]	80.75[a]	0.11
5	0.94[d]	78.44[a]	70.63[c]	76.38[b]	0.17
6	0.00[c]	75.63[a]	67.50[b]	70.00[b]	0.59
7	0.00[c]	64.38[b]	61.56[b]	69.63[a]	0.48
8	0.00[c]	63.75[b]	53.75[b]	64.69[a]	0.06
9	0.00[d]	58.44[b]	51.88[c]	62.19[a]	0.06
10	0.00[d]	56.25[b]	40.63[c]	61.88[a]	0.06
11	0.00[d]	52.50[b]	37.56[c]	56.25[a]	0.18
12	0.00[c]	44.25[ab]	37.75[b]	50.00[a]	1.09
13	0.00[c]	40.00[a]	20.00[b]	45.31[a]	1.53
14	0.00[d]	38.94[b]	15.63[c]	41.56[a]	0.05
15	0.00[d]	34.88[b]	15.31[c]	36.25[a]	0.12
16	0.00[d]	28.75[a]	13.13[c]	27.19[b]	0.02
17	0.00[c]	25.44[a]	12.81[b]	26.63[a]	0.53
18	0.00[c]	19.06[a]	12.19[b]	20.00[a]	0.57
19	0.00[c]	0.00[c]	9.06[b]	17.19[a]	0.01
20	0.00[c]	0.00[c]	5.94[b]	15.00[a]	0.54
21	0.00[c]	0.00[c]	2.19[b]	13.31[a]	0.00
22	0.00[c]	0.00[c]	1.56[b]	9.69[a]	0.01
23	0.00[b]	0.00[b]	0.21[b]	6.56[a]	0.04
24	0.00[b]	0.00[b]	0.00[b]	3.75[a]	0.01
25	0.00[b]	0.00[b]	0.00[b]	2.81[a]	0.01
26	0.00[b]	0.00[b]	0.00[b]	2.19[a]	0.00

Means with the different superscripts are significantly different ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

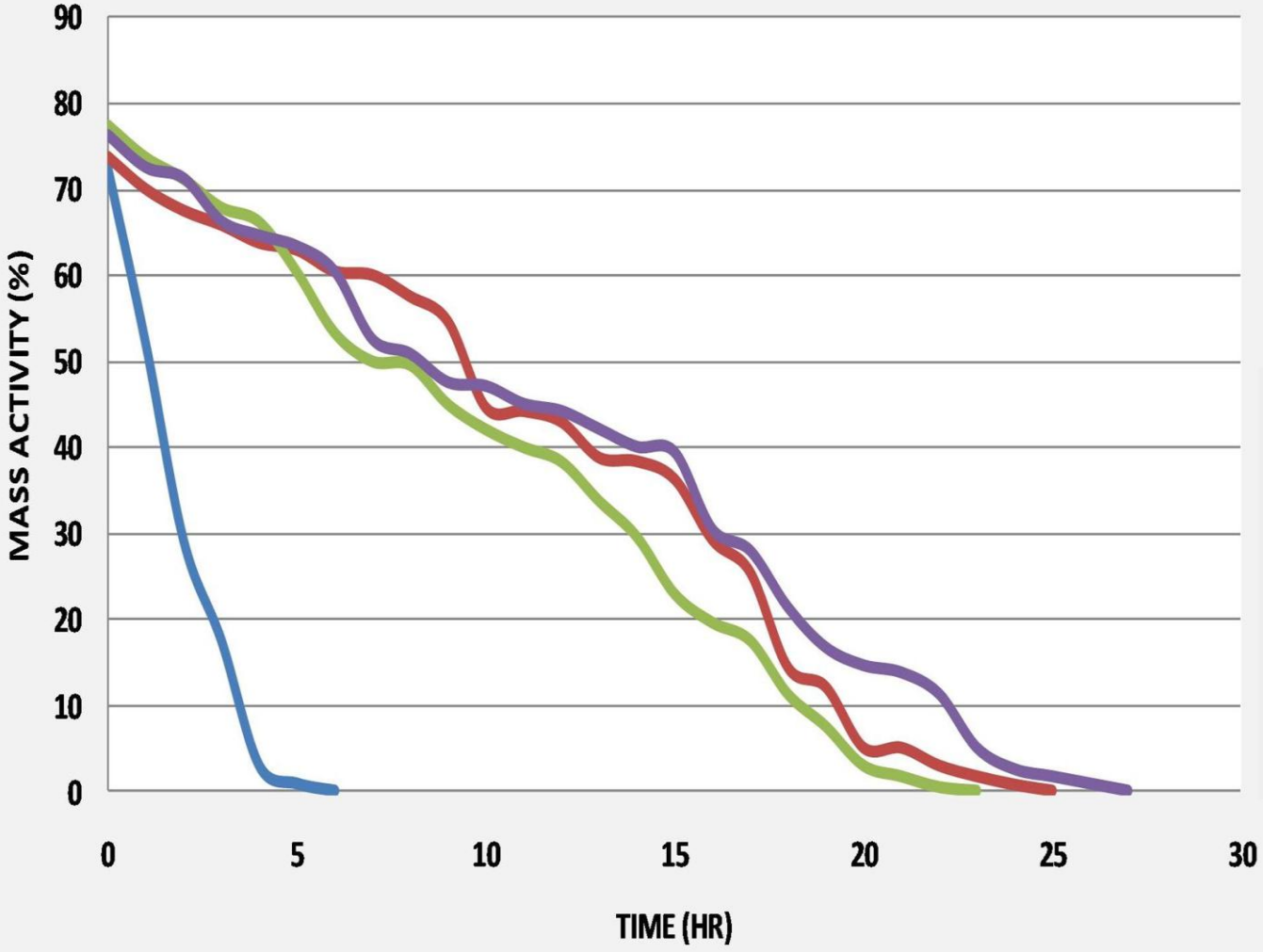
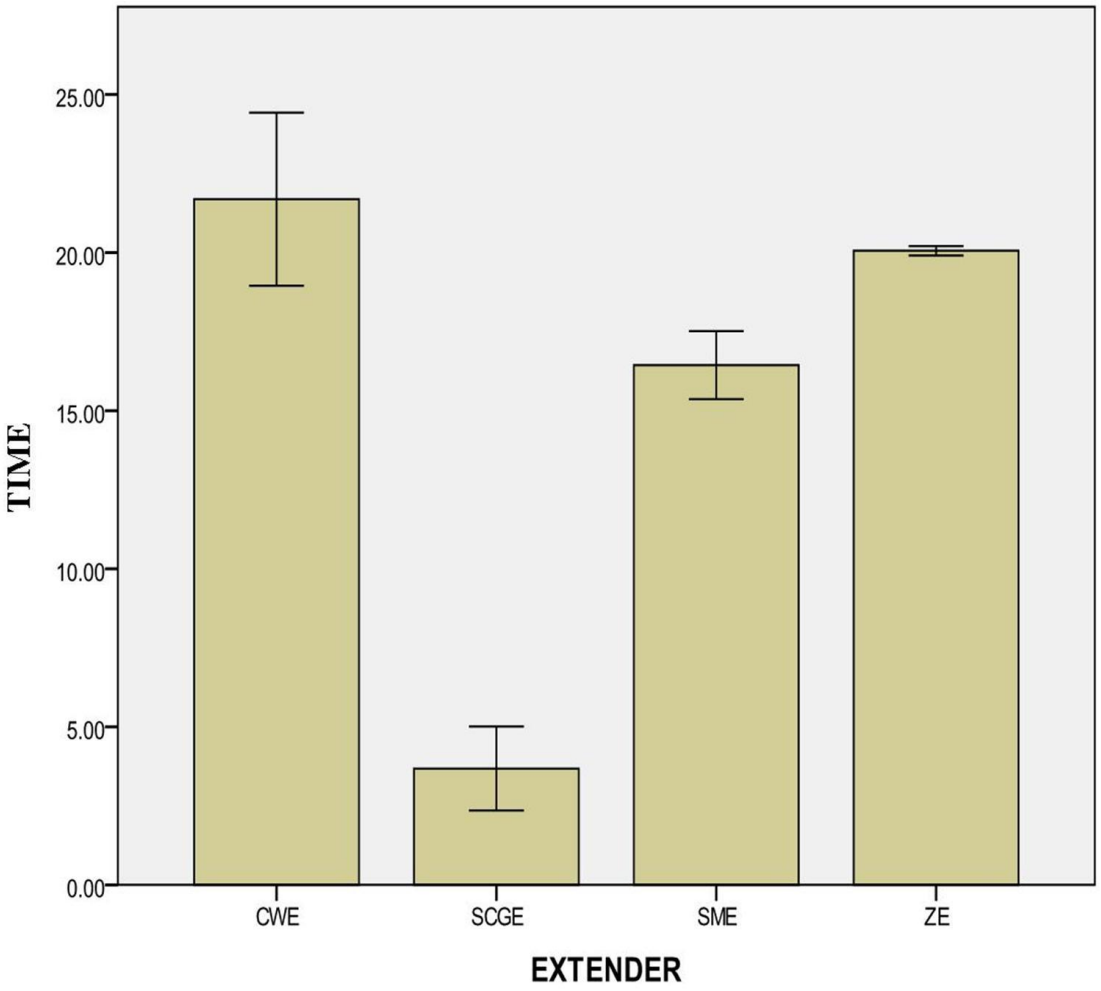


Fig. 3.2: Rate of change of Mass Activity with time of spermatozoa preserved at 28°C

Table 3.2: Mass Activity (%) of canine semen stored at room temperature (28°C)

TIME (HR)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
0	72.50^{d}	73.75^{c}	77.50^{a}	76.25^{b}	0.16
1	52.08^{b}	70.00^{a}	73.75^{a}	72.50^{a}	0.51
2	29.17^{c}	67.50^{b}	71.25^{a}	71.25^{a}	0.22
3	17.50^{c}	65.83^{b}	67.92^{a}	62.25^{b}	0.14
4	2.92^{c}	63.75^{b}	66.25^{a}	64.58^{b}	0.16
5	0.83^{d}	62.92^{b}	60.42^{c}	63.33^{a}	0.05
6	0.00^{c}	60.42^{a}	53.33^{b}	60.42^{a}	0.16
7	0.00^{c}	60.00^{a}	50.00^{b}	52.50^{b}	0.83
8	0.00^{d}	57.50^{a}	49.58^{c}	50.83^{b}	0.11
9	0.00^{c}	54.58^{a}	45.00^{b}	47.50^{b}	0.53
10	0.00^{d}	44.58^{b}	42.08^{c}	47.08^{a}	0.15
11	0.00^{c}	44.17^{ab}	40.00^{b}	45.00^{a}	0.66
12	0.00^{c}	42.92^{a}	38.33^{b}	44.17^{a}	0.17
13	0.00^{d}	38.75^{b}	33.75^{c}	42.08^{a}	0.43
14	0.00^{c}	38.33^{a}	29.58^{b}	40.00^{a}	0.61
15	0.00^{d}	36.25^{b}	22.92^{c}	39.38^{a}	0.22
16	0.00^{d}	29.17^{b}	19.58^{c}	30.41^{a}	0.05
17	0.00^{d}	25.42^{b}	17.50^{c}	27.92^{a}	0.27
18	0.00^{d}	14.33^{b}	11.25^{c}	21.33^{a}	0.21
19	0.00^{d}	12.08^{b}	7.50^{c}	16.67^{a}	0.03
20	0.00^{d}	5.00^{b}	2.92^{c}	14.58^{a}	0.12
21	0.00^{d}	5.00^{b}	1.67^{c}	13.75^{a}	0.06
22	0.00^{d}	2.92^{b}	0.42^{c}	11.25^{a}	0.01
23	0.00^{c}	1.67^{b}	0.00^{c}	5.00^{a}	0.07
24	0.00^{c}	0.70^{b}	0.00^{c}	2.50^{a}	0.05
25	0.00^{b}	0.00^{b}	0.00^{b}	1.67^{a}	0.00
26	0.00^{b}	0.00^{b}	0.00^{b}	0.83^{a}	0.00

Means with the different superscripts are significantly different ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender



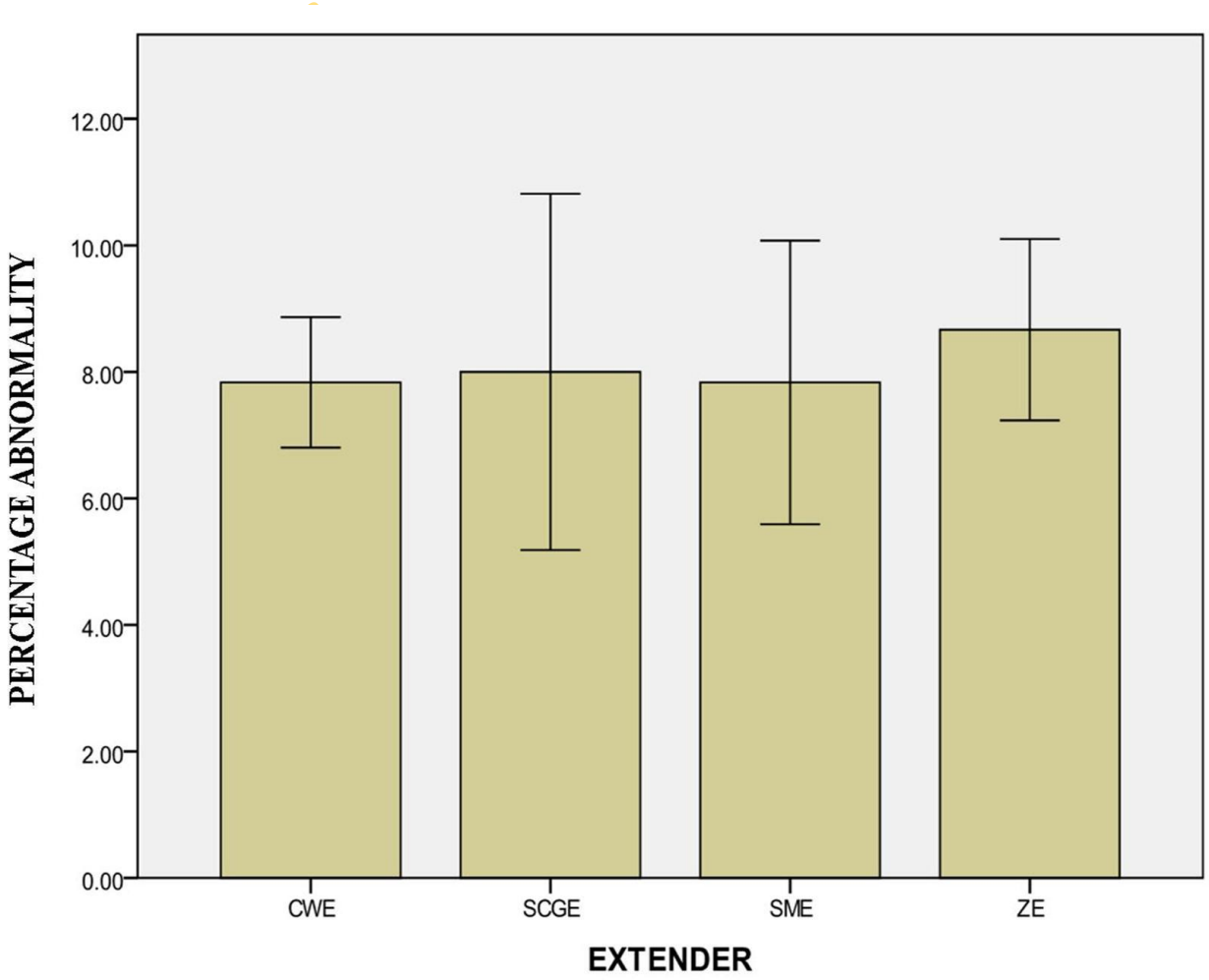
SCGE: Sodium Citrate Glycine Extender

CWE: Coconut Water Extender

SME: Skim Milk Extender

ZE: Zobo Extender

Fig. 3.3: Survival time (hr) of spermatozoa at 28°C



SCGE: Sodium Citrate Glycine Extender

CWE: Coconut Water Extender

SME: Skim Milk Extender

ZE: Zobo Extender

Fig. 3.4: Percentage abnormality (%) of spermatozoa at 28°C

SEMEN PRESERVATION AT REFRIGERATION TEMPERATURE

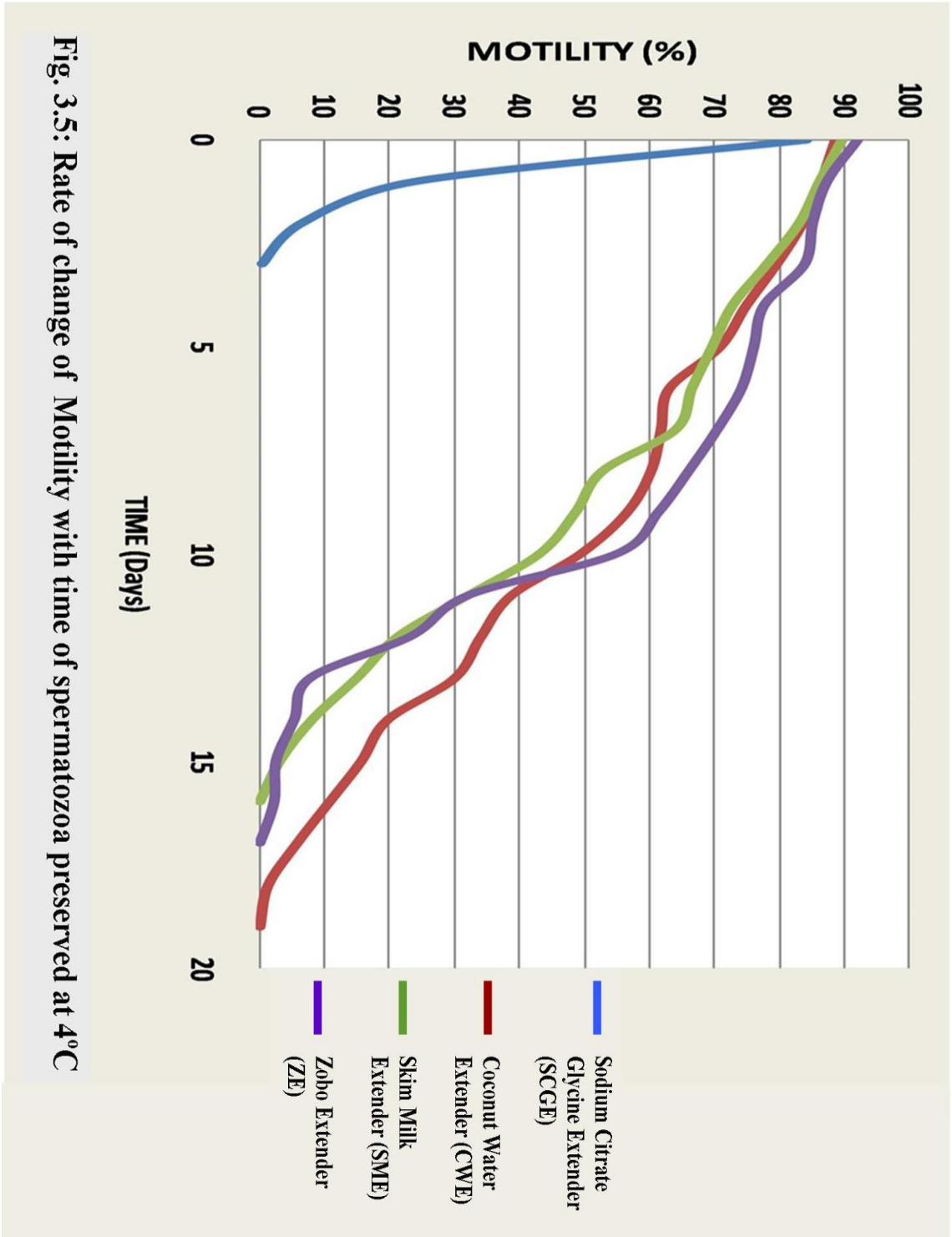


Fig. 3.5: Rate of change of Motility with time of spermatozoa preserved at 4°C

Table 3.3: Progressive Motility (%) of canine semen stored at refrigeration temperature ($4^{o}C$)

TIME (Day)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
0	84.06^{d}	88.75^{c}	89.69^{b}	91.88^{a}	0.07
1	21.04^{b}	86.56^{a}	86.26^{a}	87.19^{a}	0.19
2	6.56^{b}	83.75^{a}	83.13^{a}	85.00^{a}	0.28
3	0.31^{d}	79.69^{b}	78.13^{c}	83.75^{a}	0.03
4	0.00^{c}	75.00^{ab}	72.81^{b}	77.50^{a}	0.50
5	0.00^{d}	70.94^{b}	69.69^{c}	75.94^{a}	0.00
6	0.00^{d}	63.13^{c}	66.56^{b}	74.06^{a}	0.21
7	0.00^{d}	61.87^{c}	64.06^{b}	70.31^{a}	0.16
8	0.00^{d}	60.31^{b}	52.81^{c}	65.94^{a}	0.17
9	0.00^{d}	56.56^{b}	48.63^{c}	61.25^{a}	0.12
10	0.00^{d}	49.38^{b}	43.13^{c}	54.94^{a}	0.05
11	0.00^{c}	38.75^{a}	32.19^{b}	32.19^{b}	0.03
12	0.00^{d}	34.06^{a}	21.29^{c}	23.19^{b}	0.11
13	0.00^{d}	30.00^{a}	15.00^{b}	7.81^{c}	0.17
14	0.00^{d}	19.69^{a}	8.13^{b}	5.31^{c}	0.01
15	0.00^{d}	15.63^{a}	3.12^{b}	2.50^{c}	0.01
16	0.00^{c}	10.63^{a}	0.00^{c}	2.18^{b}	0.03
17	0.00^{b}	5.63^{a}	0.00^{b}	0.00^{b}	0.00
18	0.00^{b}	1.25^{a}	0.00^{b}	0.00^{b}	0.01

Means with the different superscripts are significantly different ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

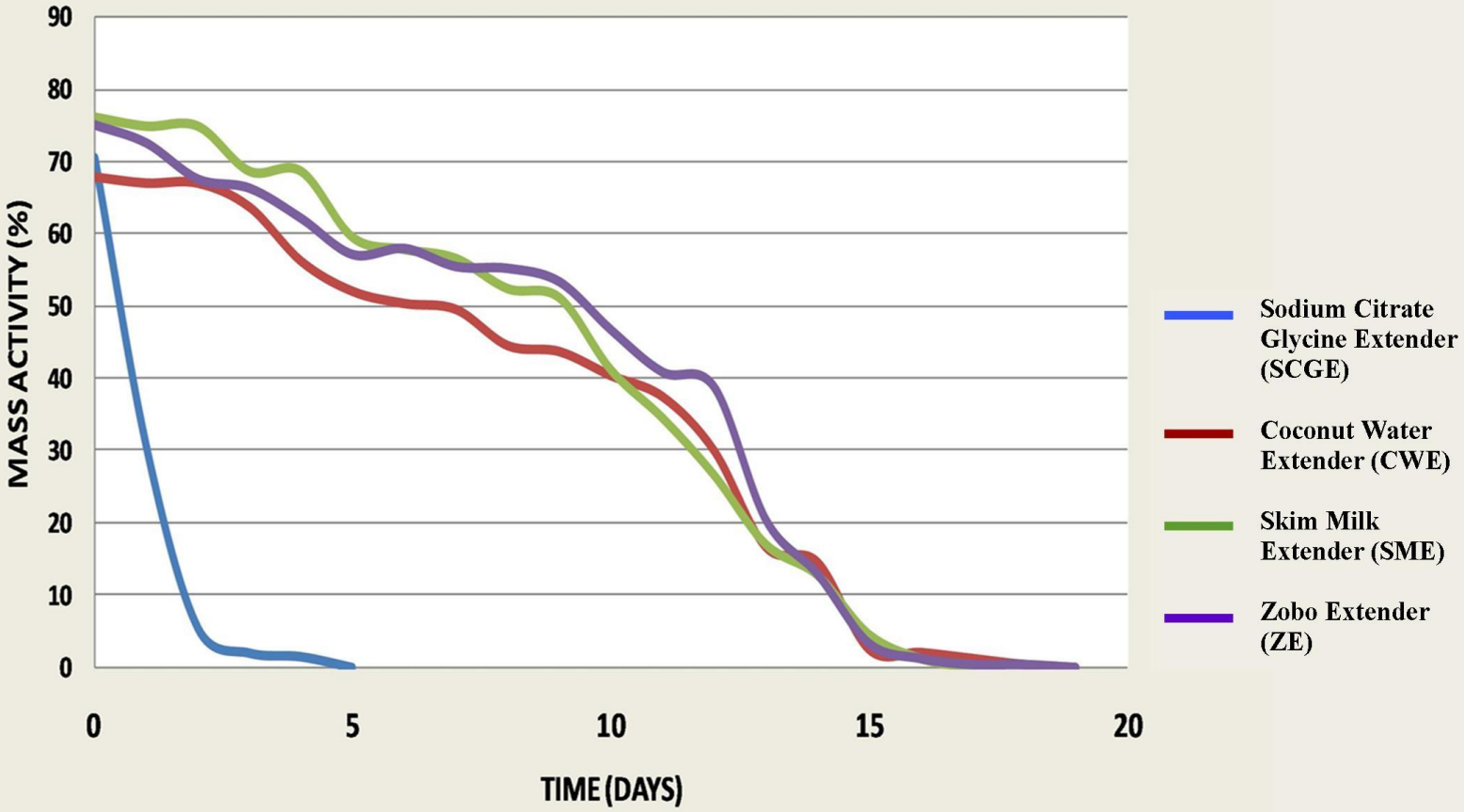


Fig. 3.6: Rate of change of Mass Activity with time of spermatozoa preserved at 4°C

Table 3.4: Mass Activity (%) of canine semen stored at room temperature (4^{o}C)

TIME (Day)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
0	70.50^{b}	67.92^{b}	76.25^{a}	75.00^{a}	0.55
1	30.63^{d}	67.08^{c}	75.00^{a}	72.50^{b}	0.30
2	5.50^{d}	67.08^{c}	75.00^{a}	67.50^{b}	0.30
3	2.00^{d}	63.75^{c}	68.75^{a}	66.25^{b}	0.15
4	1.50^{d}	56.25^{c}	68.75^{a}	62.08^{b}	0.28
5	0.00^{d}	52.08^{c}	59.58^{a}	57.08^{b}	0.23
6	0.00^{c}	50.41^{b}	57.92^{a}	57.92^{a}	0.26
7	0.00^{c}	49.58^{b}	56.67^{a}	55.42^{a}	0.67
8	0.00^{d}	44.58^{c}	52.50^{b}	55.17^{a}	0.30
9	0.00^{c}	43.75^{b}	51.25^{a}	53.33^{a}	0.33
10	0.00^{c}	40.42^{b}	41.25^{b}	46.67^{a}	0.57
11	0.00^{d}	37.50^{b}	34.58^{c}	40.83^{a}	0.34
12	0.00^{c}	30.00^{ab}	26.67^{b}	38.75^{a}	1.24
13	0.00^{d}	16.67^{c}	17.08^{bc}	20.42^{a}	0.49
14	0.00^{c}	14.58^{a}	12.83^{b}	12.92^{b}	0.20
15	0.00^{d}	2.50^{c}	4.58^{a}	3.33^{b}	0.05
16	0.00^{c}	2.08^{a}	1.25^{b}	1.25^{b}	0.04
17	0.00^{c}	1.25^{a}	0.00^{c}	0.42^{b}	0.04
18	0.00^{a}	0.42^{b}	0.00^{a}	0.42^{b}	0.05

Means with the different superscripts are significantly different ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

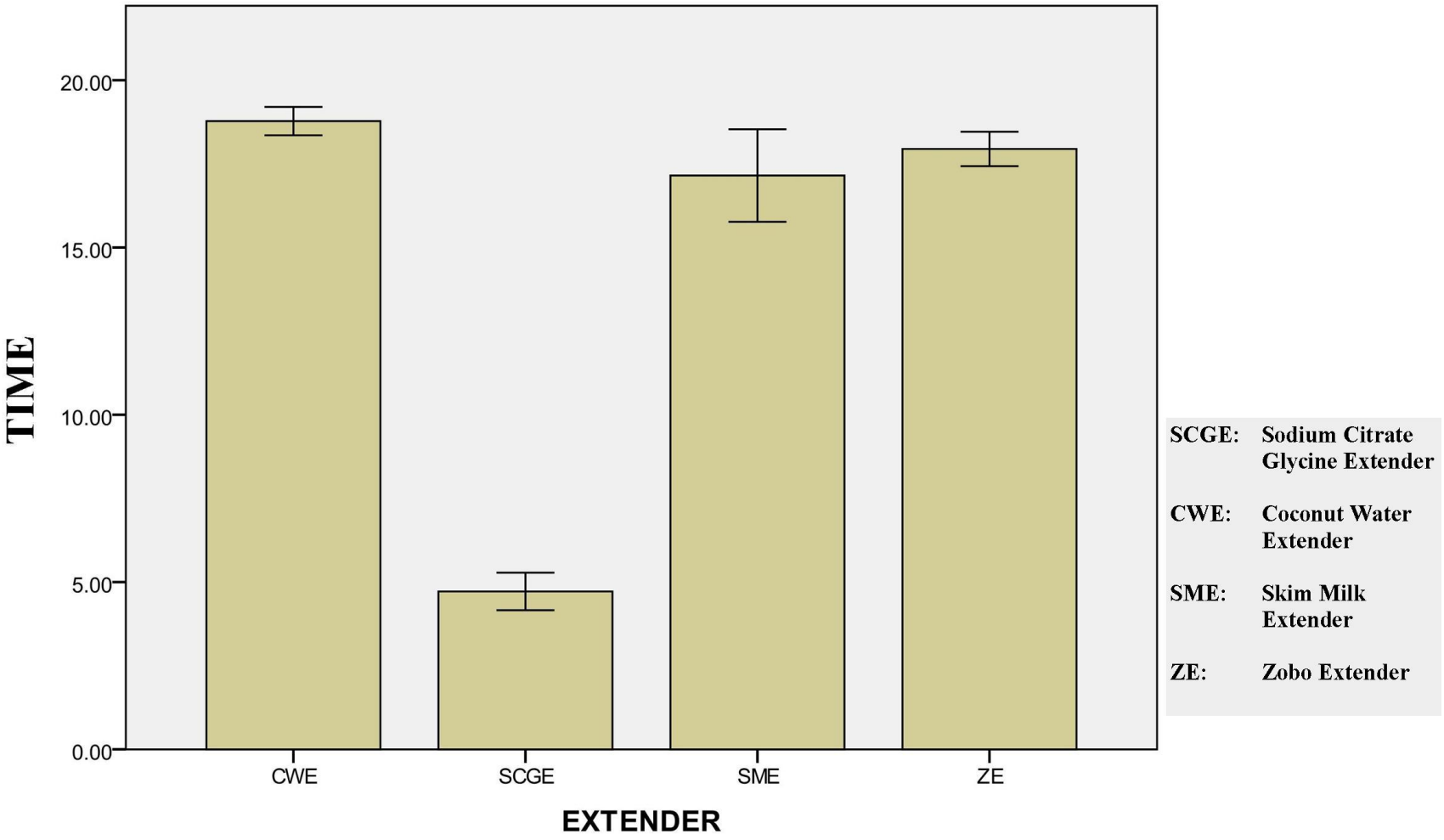


Fig. 3.7: Survival time (day) of spermatozoa at 4°C

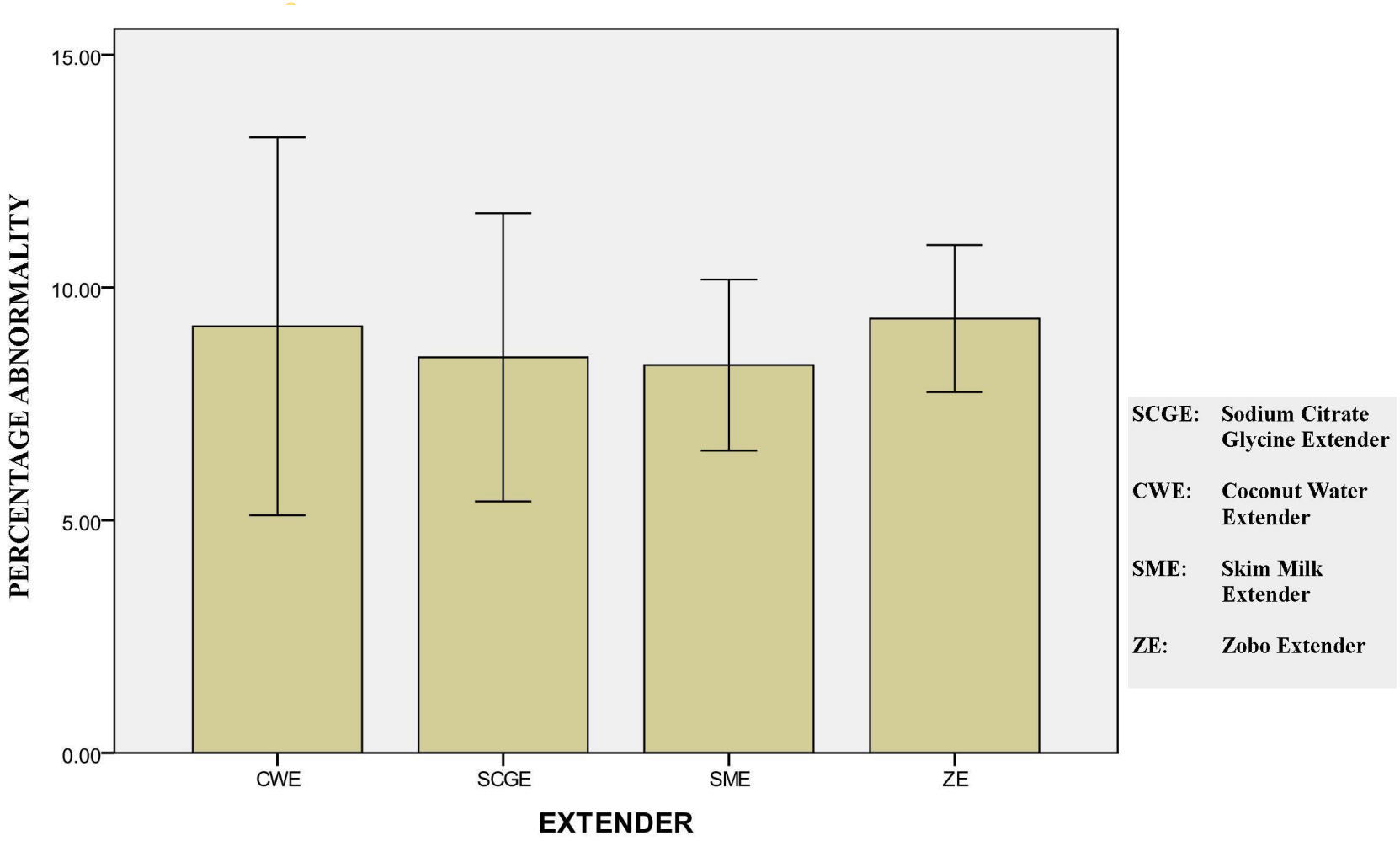


Fig. 3.8: Percentage abnormality (%) of spermatozoa at 4°C

Table 3.5: Summary of results obtained for survival time and percentage abnormalities of canine spermatozoa preserved at room temperature (28^{o}C) and refrigeration temperature (4^{o}C)

TIME (HR)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
SURVIVAL TIME (HR) at 28^{o}C	5.83^{d}	26.00^{b}	24.67^{c}	27.83^{a}	0.14
SURVIVAL TIME (DAYS) at 4^{o}C	4.72^{c}	18.78^{a}	17.15^{b}	17.95^{ab}	0.13
PERCENTAGE ABNORMALITIES at 28^{o}C	8.00^{a}	7.83^{a}	7.83^{a}	8.67^{a}	0.32
PERCENTAGE ABNORMALITIES at 4^{o}C	8.50^{a}	9.17^{a}	8.33^{a}	9.33^{a}	0.45

Means with the different superscripts are significantly different ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

DISCUSSION

3.13 SEMEN PRESERVATION AT ROOM TEMPERATURE

3.13.1 PROGRESSIVE MOTILITY

Although there were no significant differences between the initial results obtained for the Coconut Water Extender, Skim Milk Extender and the Zobo Extender (Fig. 3.1), the Zobo extender produced the highest initial progressive motility. All 4 extenders had an initial progressive motility high above the minimum recommended value.

Progressive Motility however declined at different rates with time, exposing the suitability of the various extenders for semen preservation and artificial insemination. The SCGE could only be used within the first hour and a half post extension which makes it less reliable than fresh unextended semen. Nevertheless in regards to insemination involving more than one bitch, SCGE can still be employed.

Skim Milk Extender could be kept for insemination for as long as 7hours post extension. The inability of Skim Milk Extender to match the other extenders (excluding SCGE) might be as a result of Skim Milk's poor buffering ability as there is a change for the pH of the medium to be altered with time as rancidity gradually develops in the medium.

The Coconut Water and Zobo Extenders were able to maintain semen progressive motility above the recommended minimum level (60%) for slightly over 8 and 10 hours, respectively. The impressive results obtained from the Zobo Extender may be as a result of the ascorbic acid content of Zobo which acts as a good pH stabilizer. Hence, there was a slower build up of lactic acid in the medium as a result of the metabolic processes occurring within the spermatozoa. By the 27^{th} hour, zero percent progressive motility was recorded in the Zobo Extender. This result was 4hrs longer than Skim Milk Extender and 7 hours longer than the Coconut Water Extender. This does not however suggest the superiority of Skim Milk Extender over Coconut Water Extender as though spermatozoa extended in Skim Milk Extender had a longer motility time. These results fell below the recommended minimum level from the 7^{th} hour.

3.13.2 MASS ACTIVITY

Although Zobo Extender maintained the percentage motility for slightly over 10 hours (Table 3.1) it could not match this up by maintaining the mass activity as the mass activity fell slightly below the recommended minimum by the 8th hour which reduces the possibility for fertilization to occur. Thus in reality, Zobo Extender can only be used for a maximum of 8hrs when preserving canine spermatozoa at room temperature.

It thus follows that using a combination of mass activity and progressive motility as yardsticks, Coconut Water Extender could be used for Insemination as long as 9hrs post extension while Skim Milk Extender could be used for insemination as long as 7hours post extension. Spermatozoa extended in SCGE could only be used for artificial insemination within the first hour of insemination. Coconut Water Extender is thus the extender of choice when preserving semen at room temperature or when performing delayed Artificial Insemination. This high mass activity would improve the fertilizing potential of the spermatozoa as high mass activity increases the ability of spermatozoa to penetrate the mucus membrane and also increases spermatozoa flexibility and ability of to navigate complex mucus depositions (Suarez and Osman, 1987).

High mass activity is needed for effective insemination. There is a high binding affinity between spermatozoa and the endosalpingeal epithelium. Nature might have this mechanism in place to help bind spermatozoa thus preserving them until the oocytes are released. However, this binding of the spermatozoa must be reduced in order for conception to occur. High mass activity and hyperactivation helps detach the spermatozoa from the endosalpingeal epithelium (DeMott and Suarez, 1992). Although capacitation is responsible for reducing this binding affinity, it is hyperactivitation that hastens the detachment of sperm bound to the epithelium. Hence, if insemination is to be performed immediately after extension, then Skim Milk Extender and Zobo Extenders might be preferred as semen preserved in these extenders all had significantly higher mass activity.

3.13.3 SURVIVAL TIME

That SCGE had the shorted survival time may have been due to the high mass activity (70.50% Fig. 3.2) observed coupled with the inability of the medium to guard against drastic pH change

due to a lactic acid build up in the medium. This agrees with the works of Norman *et al.* (1958) that an accumulation of lactic acid over a period of time decreased metabolic activity and subsequently survival time of spermatozoa.

The long survival time associated with the Coconut Water Extender may have been as a result of the ability of both extension media to maintain the pH of the medium. As in the case of the Zobo Extender, ascorbic acid would have been responsible for the great buffering ability of the medium. Zobo also has some antimicrobial abilities as it is used for the treatment of diseases in humans. Findings by Fullerton *et al.* (2011) indicated that sorrel was effective at all levels in inhibiting E. coli O157:H7 due to its antimicrobial ability.

Although the skim milk had citric acid as a component of its buffer, the development of acidity due to rancidity may have been too rapid for the medium to deal with. Skim Milk does have a little fat content, hence, it does go rancid when not refrigerated (Tuckey and Stadhouders, 1967).

3.13.4 PERCENTAGE ABNORMALITIES

The non significant differences between the means obtained for the percentage abnormalities of spermatozoa extended in the four different extenders (Table 3.5) follows that at both 28^{o}C and 4^{o}C, the type of extender used does not influence the percentage abnormalities of the spermatozoa. These results were not in accordance with the work of Rota *et al.* (1995), where they observed no effects of type of extender on plasma membrane or acrosome morphology of extended canine semen. However, their work was carried out at 4^{o}C. The percentage abnormalities of all four treatments fell within the recommended range as specified by (England and Heimendahl, (2010). Hence, depending on the time of insemination, all extenders could be used for Artificial Insemination in Canines.

3.14 SEMEN PRESERVATION AT REFRIGERATION TEMPERATURE

3.14.1 MOTILITY

The Sodium Citrate Glycine Extender was unable to maintain the progressive motility above the recommended minimum of 60% (Lofstedt, 2004) beyond a day. The longest time was observed in the Zobo Extender which maintained the progressive motility above 60% for as long as 9

days. This agrees with the works of Otite and Egbunike (2002). Coconut water extender was able to maintain the progressive motility of spermatozoa for a period of 8 days.

The ability of the Zobo extender to maintain the 60% minimum recommendation was probably due to the high amount of glucose associated with zobo (FAO, 1968) which provided the required energy to sustain the progressive motility of the spermatozoa, coupled with the high buffering capability associated with Zobo.

The Skim Milk Extender's ability to maintain motility above the recommended minimum for a considerable period of time at refrigeration temperature may be as a result of:

(i) The reduced activity per degree drop in temperature associated with cooling spermatozoa (Salisbury and VanDemark, 1961).

(ii) The increased shelf life of milk when refrigerated as this guards against rancidity.

This reduction in activity as a result in temperature decrease also applies to all the other extenders (with the exclusion of the Sodium citrate Glycine Extender) as the metabolic activity of spermatozoa would have been reduced at 5^{o}C thus enabling spermatozoa conserve their energy. This reduces their dependence on the nutrients of available in the extender.

In addition to the above, refrigeration reduces the effects of both harmful and spoilage bacteria as not all bacteria thrive at this temperature unlike spermatozoa preserved at room temperature.

3.14.2 MASS ACTIVITY

Not all the extenders could keep the Mass Activity of the extended spermatozoa above 50%. Hence Skim Milk and Zobo Extenders maintained the mass activity for the longest length of time (9days). Coconut Water and Sodium citrate Glycine Extenders were able to maintain the Mass Activity of extended spermatozoa for 6 days and 1 day respectively.

The results showed that the cumulative effects of mass activity and progressive motility on the fertilizing potential of canine spermatozoa, Skim Milk Extender can only be used when preserved at refrigeration temperature for a maximum of 7 days while Zobo, Sodium Citrate Glycine and Coconut Water Extenders can be used for insemination within 9, 1 and 7 days, respectively. This implies that preserving good quality semen at 5^{o}C, Zobo Extender is the

extender of choice. However, if insemination is to be done within the first day, any of the extenders would produce adequate results.

3.14.3 SURVIVAL TIME

Coconut Water had the longest survival time of 17.56 days (Fig. 3.7). The satisfactory results produced by Coconut Water, Skim Milk and Zobo Extenders might be as a result of the citric and sodium Citrate in the extenders. Though neither of these additives were added to the Zobo Extender, zobo contains a substantial amount of ascorbic acid (Table 2.1) which may have augmented the ability of the extender to keep the pH within the required range. The effects of refrigeration on reducing the rate of rancidity in skim milk could have been the main factor responsible for the long survival time of the spermatozoa.

3.14.4 PERCENTAGE ABNORMALITIES

Though the results obtained showed that the Zobo Extender had the highest number of abnormal spermatozoa (Table 3.8), these were however not significantly different from those obtained from the Skim Milk, Coconut Water and Sodium Citrate Glycine extenders. All extenders had a percentage abnormality within the accepted range of 30% (Günzel-Apel, 1994) hence, it is deduced that refrigeration has no effect on the percentage abnormality of spermatozoa provided the right extenders are used. Nevertheless, one may still credit the higher number of percentage abnormal spermatozoa observed in the Zobo Extender to its high mass activity which would have in turn increased the friction between the spermatozoa and their surrounding media. Although the Skim milk Extender showed a higher mass activity, the presence of few fat globules may have been a cushioning factor in this case. Thus, Coconut Water and Sodium Citrate Glycine Extenders had a higher percentage motility than spermatozoa extended in Skim milk Extender.

3.15 CONCLUSION

Though the percentage abnormalities were not significantly affected by both the type of extenders used as well as the two different temperatures of storage, the type of extender used for preservation as well as the preservation temperature had effect on the quality of spermatozoa. When preserving semen at room temperature, Coconut Water Extender will produce the best results.

However, if preservation is to be done at refrigeration temperature, Zobo Extender is the extender of choice.

Since the results obtained for the initial post preservation evaluation fell within the accepted range, it entails that if insemination is to be preformed immediately after extension any of the extenders is suitable as long as the bitch is inseminated at the optimum time.

CHAPTER FOUR

EXPERIMENT 2

CRYOPRESERVATION OF CANINE SEMEN

4.1 INTRODUCTION

The use of distant strains of dog breeds for reproduction in Nigeria can be enhanced through the adoption of more scientific methods of reproduction. Transportation of frozen semen is one means of utilizing proven studs with desired traits for breeding purposes. This does not only reduce the huge cost associated with transporting large animals but also increases the availability of the stud. Semen can be flown in from any part of the world for breeding or research purposes. This has resulted in a new form of business in the canine industry with numerous companies specializing in sperm banks or accessories.

The need to adopt such methods does not apply to the Nigerian canine industry alone, but to the entire animal production industry in Nigeria. Nigerian Animal breeds are usually of lower genetic potential largely due to the little amount of time and money injected in this area of specialized animal production in the country.

It is therefore obvious that there lies a need for the ideal extender for preserving and transporting canine semen. Such an extender must be able to withstand the rigours of transportation by maintaining the pH of the medium as well as providing essential nutrients needed to sustain the spermatozoa.

This chapter looks into the ability of 4 different extenders to maintain the quality of canine spermatozoa preserved at sub zero temperature.

4.2 MATERIALS AND METHODS

4.2.1 Experimental dogs

This was as described in section 3.2 (page 58).

4.2.2 Collection of semen

Semen samples were collected using the artificial balloon vagina (Otite and Egbunike, 2000) as described under section 3.3 (page 57).

4.2.3 Semen evaluation

Semen was evaluated for volume, concentration, mass activity, progressive motility, morphology, pH, acrosome integrity and DNA as described in section 3.4 (pages 57-60).

4.2.3.1 Acrosome integrity: : the percentage spermatozoa with compromised acrosomes was determined by placing a drop of semen on a slide and then staining with flourescein isothiocyanate conjugated peanut agglutinin staining (FIC-PNA) and the proportion of spermatozoa which absorbed the dye recorded (Szasz *et al.*, 2000). Spermatozoa was stained for 90 seconds then smeared on a glass slide and allowed to air-dry. A minimum of 100 spermatozoa were examined by light microscopy (x 630). Cells were then categorised as having: (1) An intact acrosome whereby the acrosome membrane was stained blue and remained in contact with the sperm head , (2) A damaged acrosome: whereby the membrane was damaged or separated from the sperm head and (3) A missing acrosome.

4.2.3.2 DNA Integrity: This was done by staining samples of the extended semen with acridine orange as modified by Saikhun *et al.* (2007). Spermatozoa which had their DNA denatured produced a red fluorescence, while the double-stranded DNA (native) produced green fluorescence.

4.2.4 Semen Extenders

Portions of the collected semen samples were preserved using 4 different extenders namely:

(i) Sodium-Citrate-Glycine Buffer and Extender (SCGE).
(ii) Coconut Water Extender (CWE).
(iii) Skim milk Extender (SME).

(iv) Zobo Extender (ZE).

4.2.5 Semen Extension

The collected semen was warmed alongside the prepared extender to body temperature (37^{o}C) and both were mixed together by gently inverting the mixture. Extension was done at a rate of 100 million spermatozoa to 1ml of extender.

4.2.6 Addition of Penicillin and Streptomycin

Penicillin and streptomycin were added to all the extenders at a dosage of 10^5 i.u./1000ml and 100.00mg/100ml, respectively (Otite, 2000).

4.2.7 Cryopreservation

Freezing of the extended semen to -196^{o}C was done as described by Herman *et al.* (1994). The extended semen was cooled to refrigeration temperature (5°C) by placing in a refrigerator and left to stand for (2 hours). At the same time, glycerol was mixed with a portion of the extender and placed in the refrigerator. Straws were also placed in the refrigerator in order to bring to the same temperature as the extended semen and glycerol extender mixture.

After 2 hours, the cooled glycerol extender mixture was gradually mixed with the extended semen and the mixture was later put into straws and left to equilibrate for another 4hrs. This mixture was held in the liquid nitrogen vapour for 5 minutes (England and Lofstedt, 2000) and then lowered into the liquid Nitrogen tank for complete immersion in liquid nitrogen.

4.2.8 Thawing

Thawing was done by placing the frozen semen samples in a water bath at 37^{o}C prior to evaluation.

4.2.9 Post preservation analysis

All the frozen samples were later thawed prior to analysis. Analysis included mass activity, motility, concentration, pH, live/dead ratio and acrosome integrity.

4.2.10 Statistical analysis

The results obtained where analyzed for statistical differences using the analysis of variance ($p<0.05$) and their means were subjected to Duncan's Multiple Range Test where applicable using SAS (2008).

4.3 RESULTS

The results revealed that Skim Milk Extender (SME) had the highest initial post thaw progressive motility of 71.17% (Table 4.1). This was followied by Zobo Extender (ZE), Coconut Water Extender (CWE) and Sodium Citrate Glycine Extender (SCGE) with 68.83%, 65.25% and 30.92% respectively. There were no significant differences between the results obtained except for SCGE.

The ZE had the highest initial post thaw mass activity of 60% (Table 4.2). These was followed by SME (59.17%) CWE (46.67%) and SCGE (26.25%) The results obtained for ZE did not differ significantly from the means of SME but differed from CWE and SCGE.

The effects of Cryopreservation on the survival time of spermatozoa showed that SME had significantly highest survival time of 7.67hrs (Table 4.3). ZE and SWE had survival time of 5.83 and 5.61 hrs respectively. However, these results did not differ significantly. The shortest time was observed in SCGE.

The highest percentage abnormality was recorded in SCGE (15.29%). This result did not differ significantly from CWE (13.67%) but did differ significantly from SME (9.83%) and ZE (11.21%) (Table 4.3).

The ZE had the highest percentage of compromised acrosomes (8.54%). These results were significantly higher than those obtained for CWE (6.38%), SCGE (4.80%) and SME (3.71%). There were no significant differences between the means of SCGE and CWE.

The SCGE recorded the highest denatured DNA (1.92%). These figures were significantly higher than those of CWE (1.79%), SME (1.67%) and ZE (1.67%). The results recorded for SME, CWE and ZE did not differ significantly.

A comparison between the DNA and acrosome integrity (Fig. 4.7) revealed that the acrosome of the canine spermatozoa has a 77% chance of being compromised as compared to 23% for the DNA.

Comparing the effects of preservation temperatures on the percentage abnormalities of spermatozoa (Table 4.4), the results revealed that cryopreservation significantly affected the percentage abnormalities of spermatozoa.

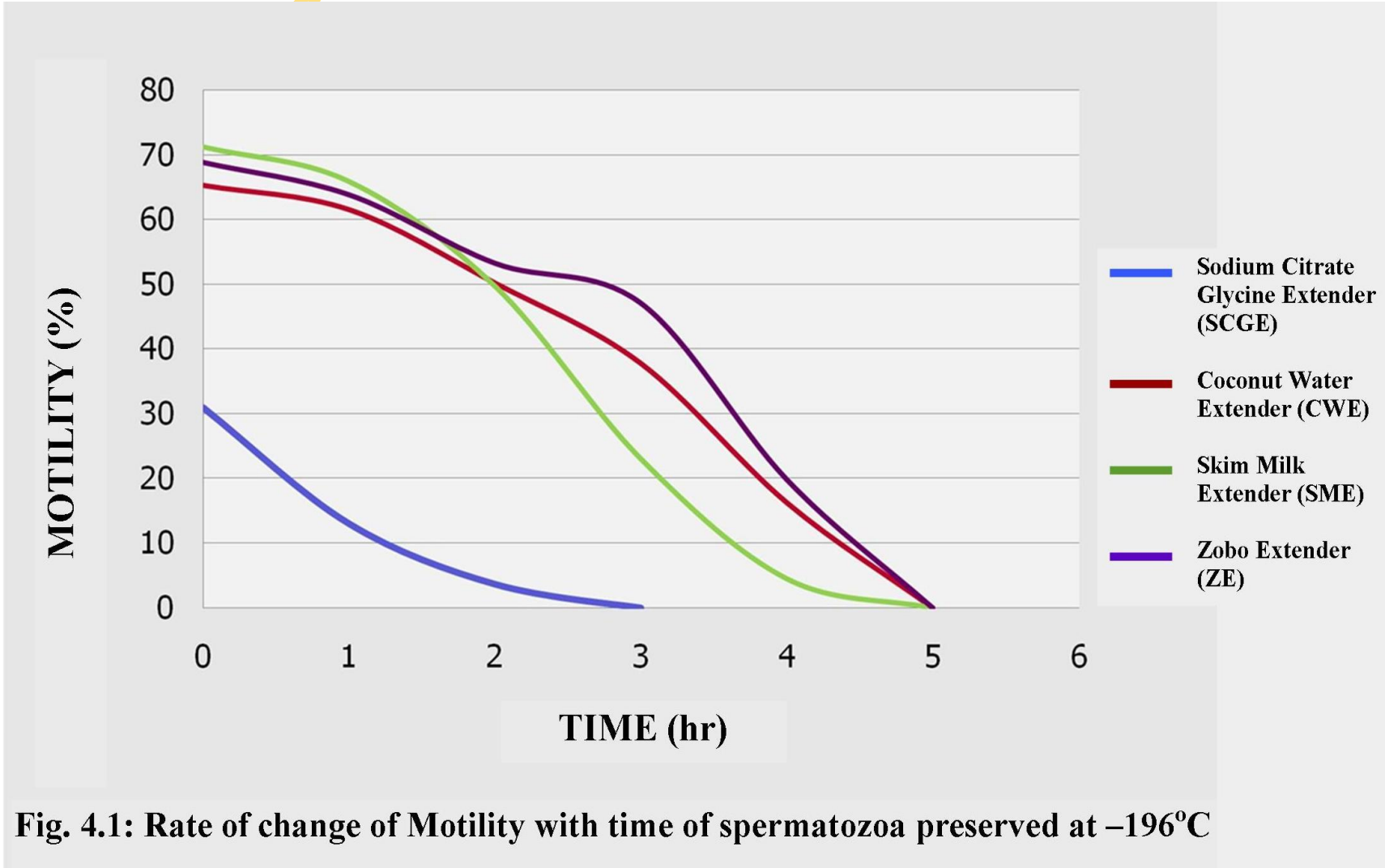


Fig. 4.1: Rate of change of Motility with time of spermatozoa preserved at –196°C

Table 4.1: Motility (%) of spermatozoa in four different extenders

TIME (Hr)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
1	30.92^{b}	65.25^{a}	71.17^{a}	68.83^{a}	1.12
2	13.00^{b}	61.50^{a}	65.83^{a}	63.83^{a}	1.12
3	3.67^{b}	50.17^{a}	49.67^{a}	53.25^{a}	0.80
4	0.00^{c}	37.35^{b}	46.92^{a}	47.08^{a}	0.64
5	0.00^{c}	16.25^{b}	23.00^{a}	19.33^{ab}	0.77
6	0.00^{b}	0.00^{b}	6.17^{a}	0.00^{b}	0.36

Means on the same row with different superscripts differ significantly (P<0.05).
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

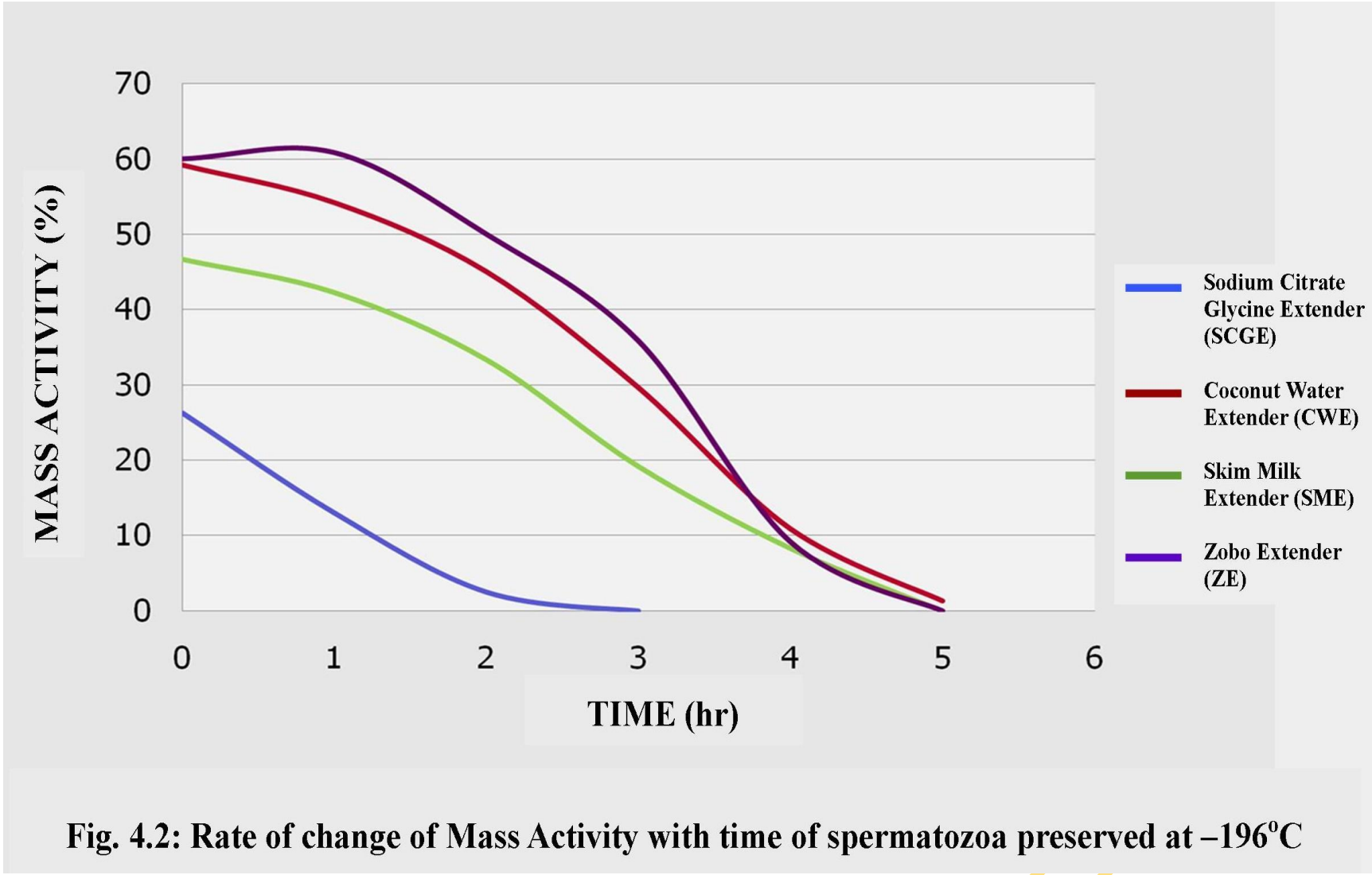


Fig. 4.2: Rate of change of Mass Activity with time of spermatozoa preserved at –196°C

Table 4.2: Mass Activity (%) of spermatozoa in four different extenders

TIME (Hr)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
1	26.25^{c}	46.67^{b}	59.17^{a}	60.00^{a}	1.24
2	13.00^{c}	42.50^{b}	54.17^{a}	60.83^{a}	1.51
3	2.50^{c}	33.33^{b}	45.00^{a}	50.00^{a}	1.09
4	0.00^{c}	23.33^{b}	29.58^{ab}	35.83^{a}	1.15
5	0.00^{b}	8.33^{a}	10.83^{a}	9.17^{a}	0.90
6	0.00^{b}	0.00^{b}	1.25^{a}	0.00^{b}	0.11

Means on the same row with different superscripts differ significantly (P<0.05).
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

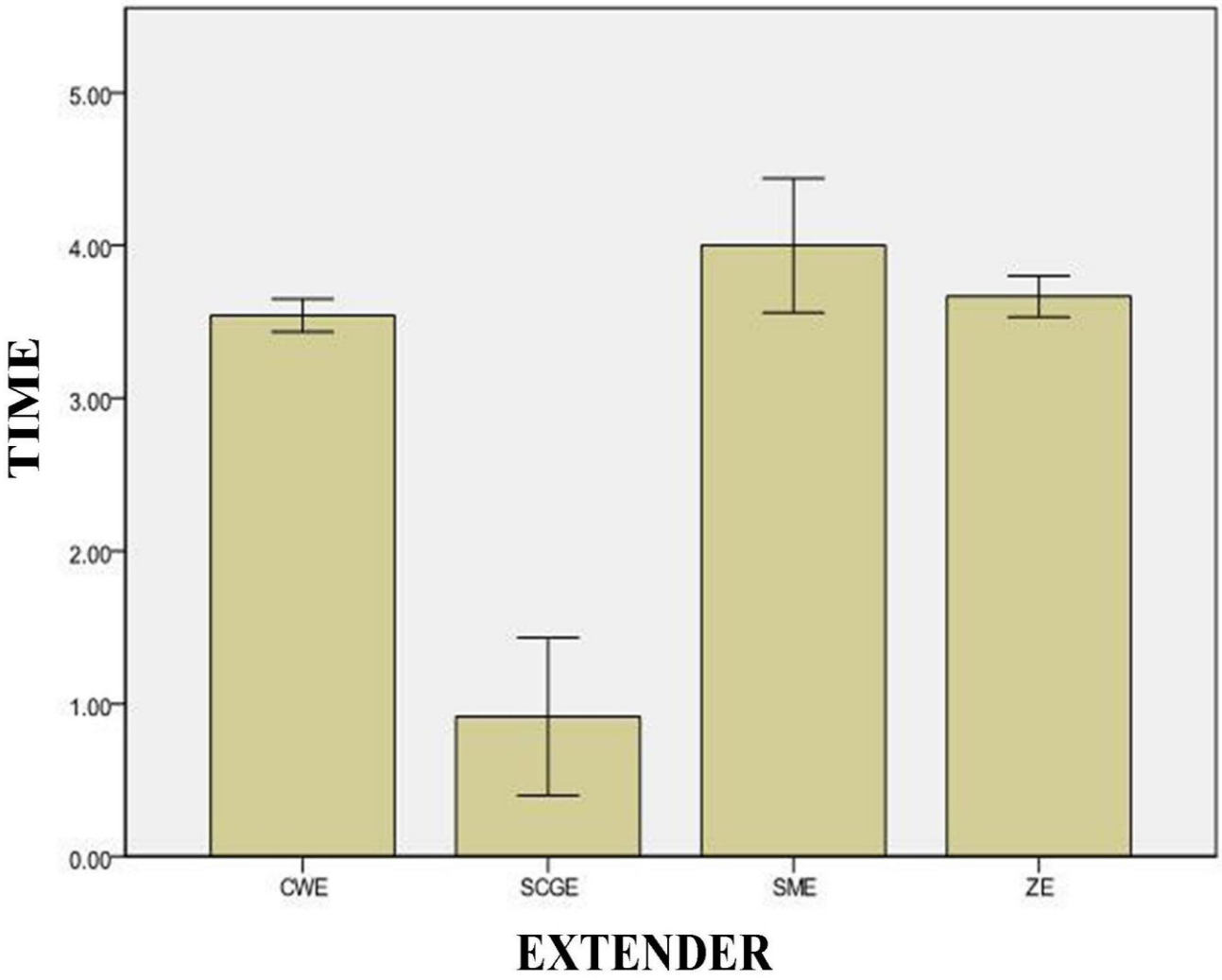


Fig. 4.3: Survival time (hr) of spermatozoa at –196^{0}C

Table 4.3: Summary of results

PARAMETERS	EXTENDERS				SEM
	SCGE	CWE	SME	ZE	
% Abnormality	15.29[a]	13.67[a]	9.83[b]	11.21[b]	0.24
% Acrosome Integrity	4.80[b]	6.38[b]	3.71[c]	8.54 [a]	0.26
% DNA Integrity	1.92[a]	1.79[bc]	1.67[c]	1.67[c]	0.26
Post Thaw survival time (hr)	3.67[c]	5.61[b]	7.67[a]	5.83[b]	0.08

Means on the same row with different superscripts differ significantly (P<0.05).
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

Table 4.4: Effects of preservation temperature on percentage abnormality of canine spermatozoa

EXTENDER	PERCENTAGE ABNORMALITY			SEM
	28°C	4°C	-196°C	
SCGE	8.00[b]	8.50[b]	15.29[a]	0.42
CWE	7.83[b]	9.17[b]	13.67[a]	0.44
SME	7.83[a]	8.33[a]	9.83[a]	0.27
ZE	8.67[b]	9.33[b]	11.21[a]	0.21

Means on the same row with different superscripts differ significantly (P<0.05).
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

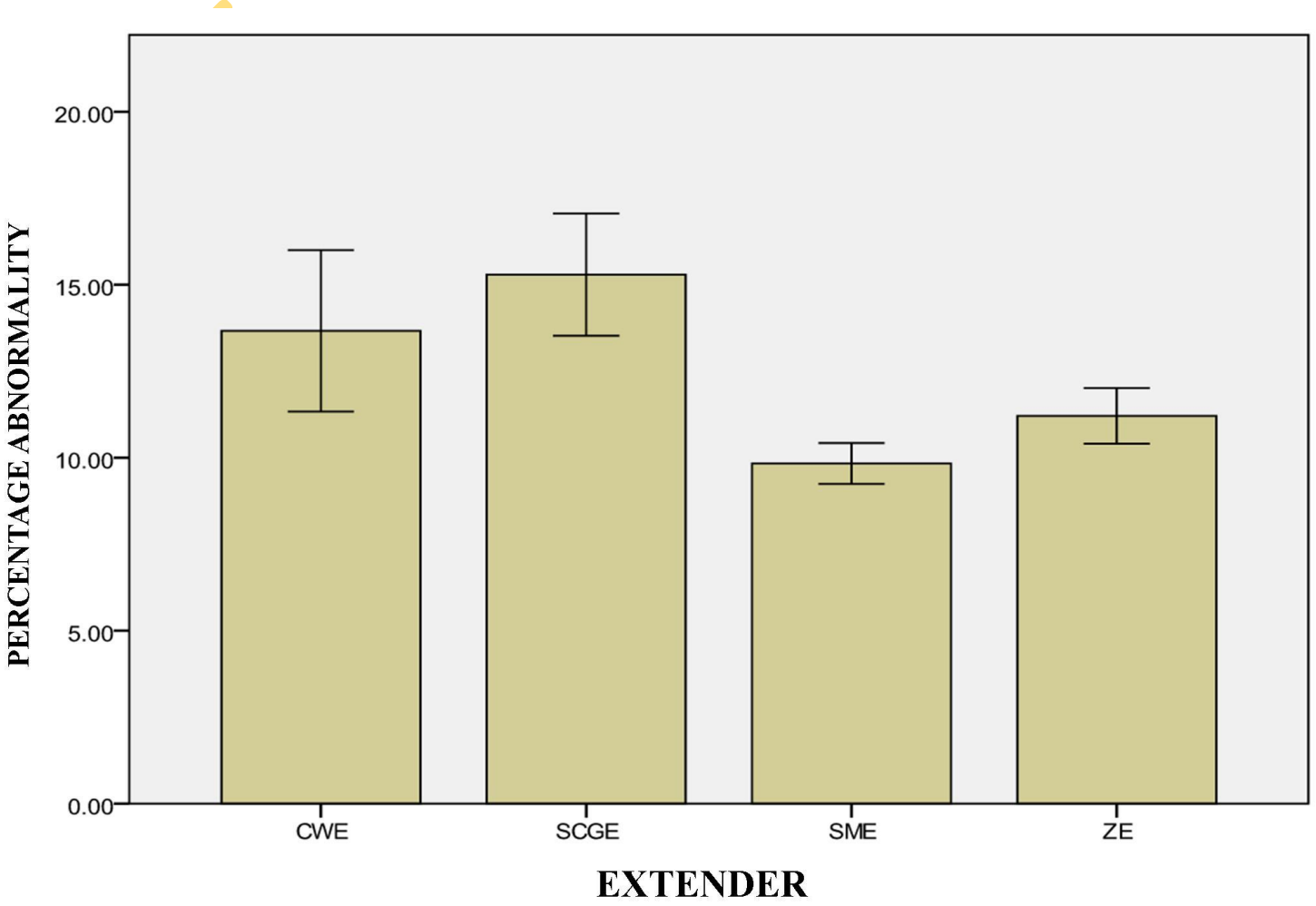


Fig. 4.4: Average percentage (%) abnormality of spermatozoa at –196°C

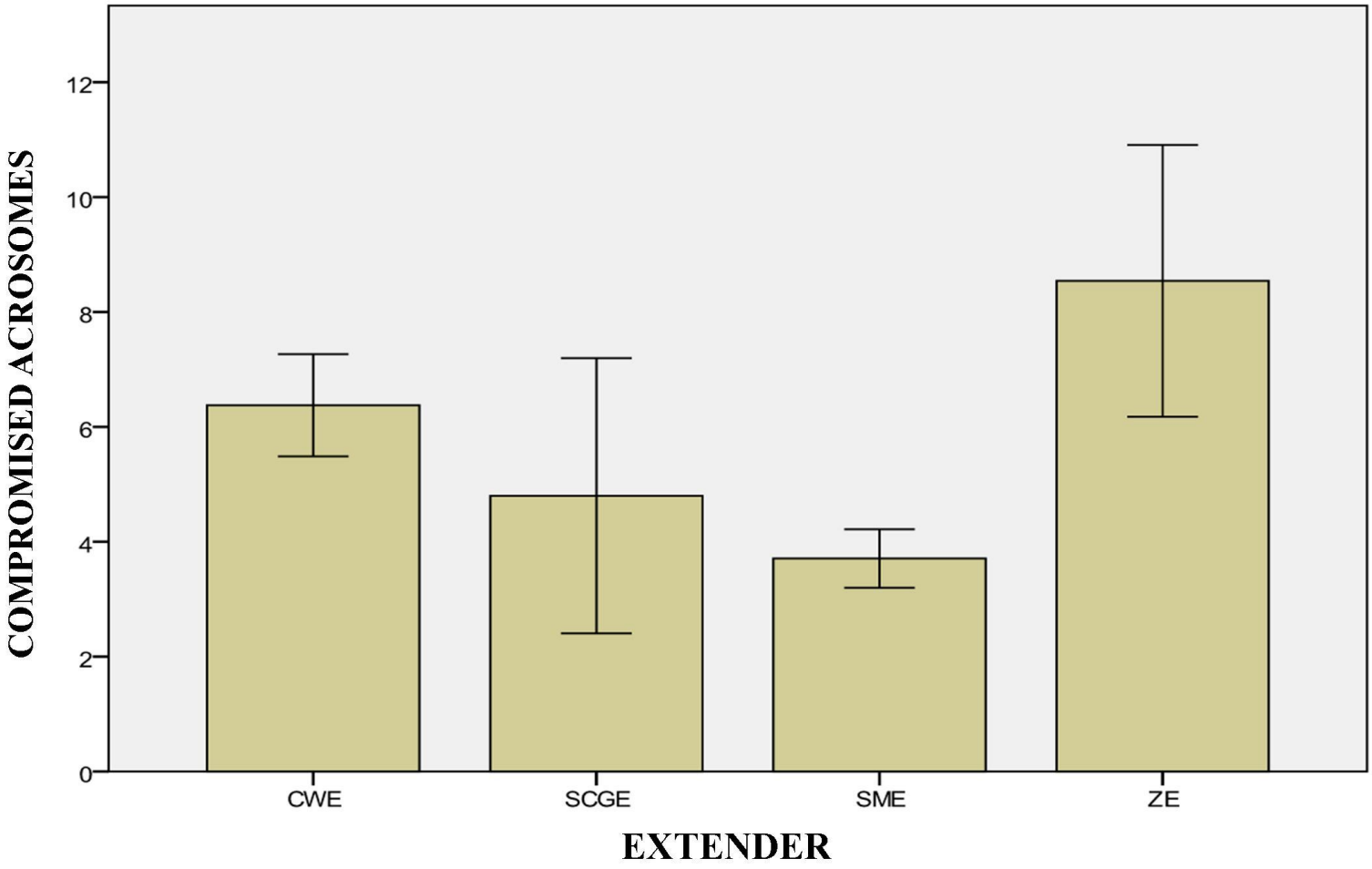


Fig. 4.5: Average percentage (%) compromised acrosomes of spermatozoa at –196°C

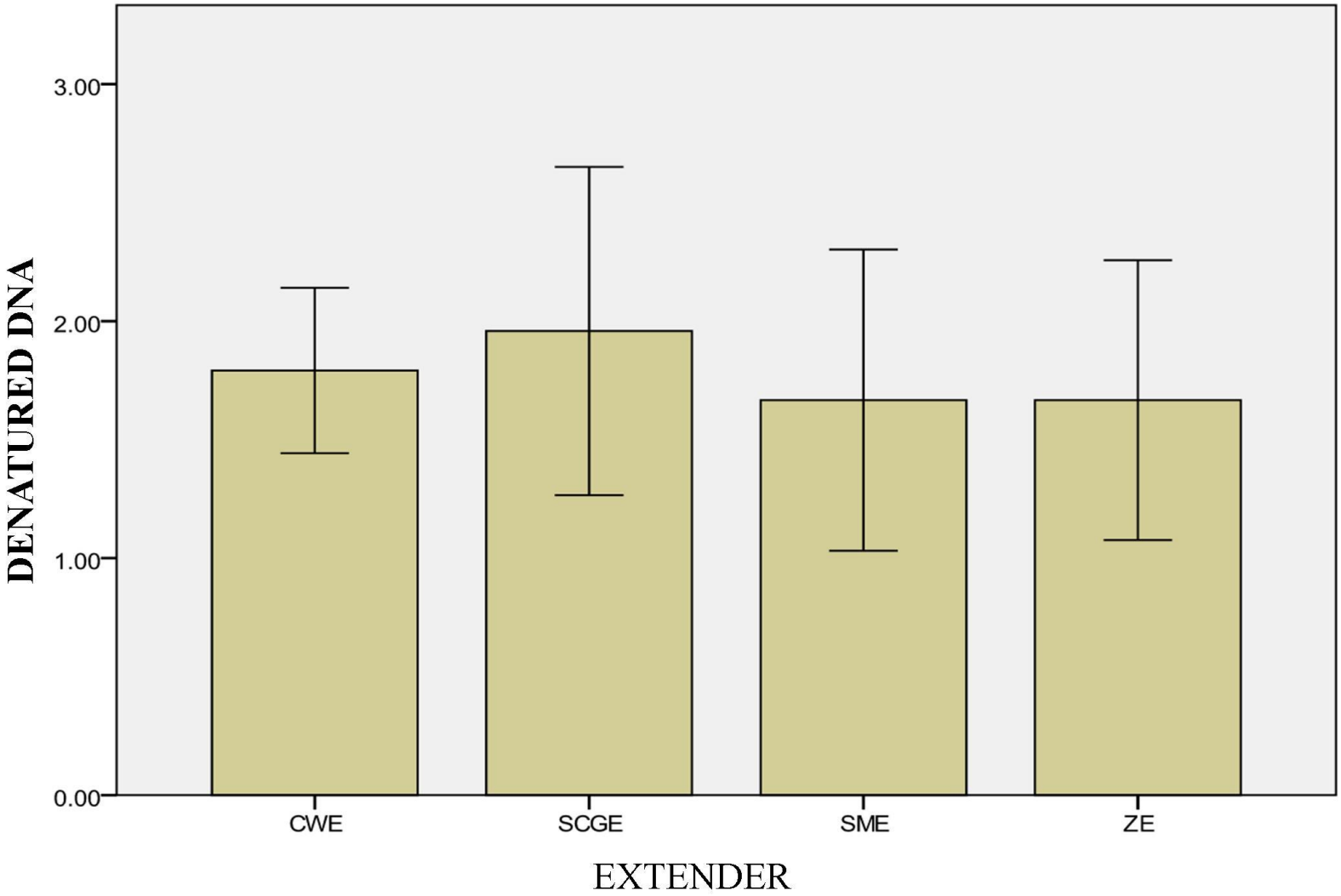


Fig. 4.6: Average percentage (%) denatured DNA of spermatozoa at –196^{o}C

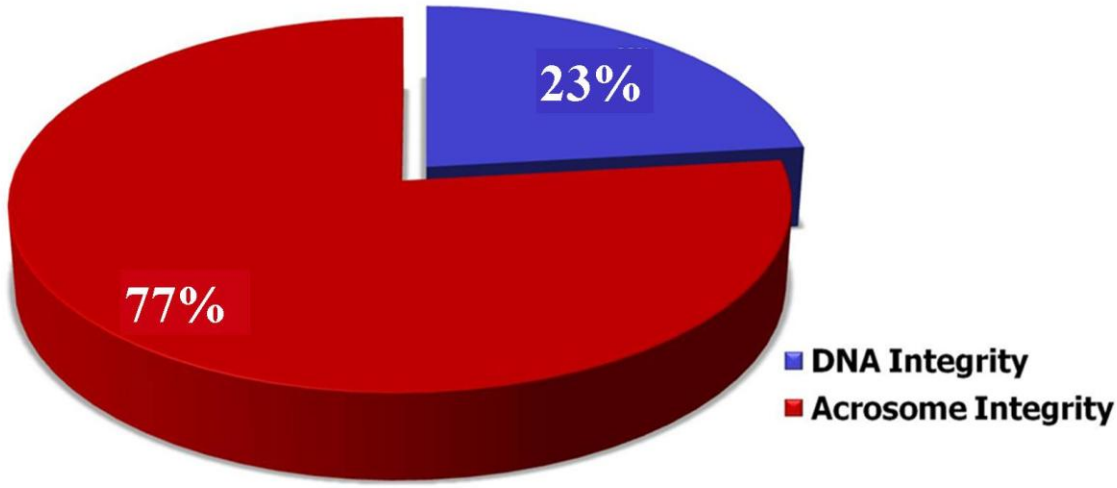


Fig 4.7: A comparison of the effects of cryopreservation on DNA integrity and acrosome integrity.

DISCUSSION

4.4.1 PROGRESSIVE MOTILITY

The high progressive motility associated with Skim Milk Extender (Table 4.1) signified the high preservatory abilities associated with skim milk. Skim milk is rich in lipoproteins which reduce the effects of cold shock hence, more motile spermatozoa were observed in this medium. All the top three extenders (Skim Milk Extender (71.17%), Zobo Extender (68.83%) and Coconut Water Extender (65.25%)) proved to have motility above the recommended minimum of 60% (Lofstedt, 2004). This tallies with the results of England and Heimendahl (2010) that good quality thawed semen should have motility ranging from 60-70%. The results obtained for Skim Milk Extender were above those giving by Crusco dos Santos *et al.* (1999) who recorded a progressive motility of 3.98% but fell within the range of Takeishi *et al.* (1976) who observed a progressive motility as high as 70% for spermatozoa preserved in Skim Milk Extender.

It was however observed that SCGE did not meet the 60% minimum requirement for progressive motility recommended for successful artificial insemination (Lofstedt, 2004). This low value (30.92%) associated with SCGE does not mean however, that semen preserved in SCGE could not be used to produce offspring as the results proved that there were still a few motile spermatozoa in the medium. With recent development and the advent of Intra Cytoplasmic Sperm Injection (ICSI) it has shown that with zero percent motility, one can still have conception as the spermatozoa are injected directly into the egg (Elder and Dale, 2011). Froman *et al.* (1984) have recorded pregnancies from inseminations carried out using semen with as low as 35-40% progressive motility.

Unlike other preservation temperatures, when considering cryopreservation, the ability to maintain pH as well as provide nutrients are less important than the ability of the medium to resist the effects of cold shock on the spermatozoa. Skim milk had the highest ability to reduce the effects of cold shock. This was probably due to the fact that skim milk contains lipoproteins which help prevent the spermatozoa against cold shock (Kiso *et al.*, 2011). These results did not differ significantly from those obtained using Coconut Water and Zobo Extender. The high results obtained from the Coconut Water and Zobo extender might be as a result of the glycerol and egg yolk added coupled with their ability to maintain stability in the medium during the

initial introduction of spermatozoa to the extender. With regards to the coconut water extender, the ability to rejuvenate the spermatozoa by resuspending them in fresh relatively alkaline coconut milk diluents might be a possibility as Norman *et al.* (1958) observed that resuspending spermatozoa in coconut milk diluents resumed metabolic activity after 6 days of storage. To date, no such work has been done on Zobo.

Since the post thaw life span of spermatozoa is limited to a few hours in the bitch (Threlfell, 2000), one might consider the ability of the extender to maintain high motility during this period as the next most important factor in determining the efficiency of an extender. Although the results obtained during the first 3 hours of post thaw analysis revealed no significant differences between Coconut water, Skim Milk Extender and Zobo Extender (Table 5.1), it however showed that the results obtained with Zobo Extender was able to prevent a massive drop in progressive motility better than the other three extenders. These results are so, probably by the fact that Zobo is rich in highly available anti oxidants such as ascorbic acid, ß-carotene and phenolic compounds, especially the anthocyanins, which further enhanced the preservatory ability of the medium (Tee *et al.*, 2002).

Although the first 3 hours results showed that the Skim milk Extender was the least capable of preventing a fast drop in progressive motility, it later picked up after the 5^{th} and 6^{th} hour (Table 4.1). This result was however not useful as the value fell far below the recommended minimum value needed for effective artificial insemination.

4.4.2 MASS ACTIVITY

The results for mass activity obtained from the initial post thaw analysis revealed that Skim Milk Extender and Zobo Extender produced the highest Mass Activity. These results were significantly higher than the results obtained from Sodium Citrate Glycine Extender and coconut Water Extender. Zobo Extender had the highest value though not significantly higher than the results of Skim Milk Extender. This high value associated with the Zobo Extender might have been due to the high amount of energy associated with the Zobo Extender as zobo is rich in glucose (FAO, 1968). This high energy associated with zobo coupled with its richness in antioxidants, which are useful in maintaining the pH of the medium must have been responsible for sustaining the mass activity of the spermatozoa above the recommended minimum of 50%.

Once semen has been thawed, the buffering ability of the extender is of paramount importance. Hence, by the third hour, the mass activity of the spermatozoa preserved in Zobo Extender had dropped to 50% which was significantly higher than the results obtained from Coconut Water Extender and Sodium Citrate Glycine Extender but not significantly higher than that obtained from Skim milk Extender. Since artificial insemination using frozen semen is performed immediately after thawing, it then follows that since the Skim milk Extender and the Zobo Extender both had their Mass Activity within the recommended range, the Skim Milk would be the superior extender as it has more motile spermatozoa than the Zobo Extender (Table 4.1, 4.2)

4.4.3 SURVIVAL TIME

The results obtained from the experiment showed indicated that the SCGE might have been detrimental to the life of spermatozoa in cryopreservation. The osmotic balance of the preservation medium is of great importance in semen preservation (Watson and Martin, 1975). While there exists a school of thought which proclaims the superiority of isotonic diluents, there also exists an opposing school of thought clamouring for the use of a hypertonic diluents in cryopreservation.

The need to draw water out of the cell (by hypertonic diluents) is of utmost importance in cryopreservation as this prevents the build up of ice crystals within the spermatozoa (Kundu *et al.*, 2002). Besides the inclusion of glycerol to the extenders prior to cryopreservation, the Zobo and Skim Milk Extenders are rich in glucose which would invariably affect the osmotic balance of the medium as sugar tends to draw water from a region of lower concentration to one of higher concentration (Starr *et al.*, 2009).

Although the results obtained for both the Skim Milk and Zobo Extenders were not significantly different, the higher value observed with the Skim Milk Extender might have been due to the fact that zobo contains less fat than milk (Otite, 2000) and hence would be less capable of preventing the effects of cold shock and hence some of the spermatozoa might have died as a result of the freezing and thawing process.

The significant superiority of the Coconut Water, Skim Milk and Zobo Extenders over their Sodium Citrate Glycine counterpart is agreement with the works of Otite (2000) who indicated the superiority of organic based extenders over their inorganic counterparts.

4.4.4 PERCENTAGE ABNORMALITY

The results showed that the Sodium Citrate Glycine was significantly highest in percentage abnormalities (15.29%). Although this fell within the accepted range of (30%) (Günzel-Apel, 1994), this must have been as a result of the rapid expansion and contraction of the spermatozoa due to the build up of ice crystals within the cell. A study of the anonymous expansion of water reveals that unlike other substances, when water freezes, it expands by one tenth (1/10) of its volume (Newell, 1903). This would result in stretching of the cell wall of the spermatozoa. As a result, numerous forms of abnormalities would occur. The issue of detached head caps which was largely observed in abnormal spermatozoa could thus be as a result of the head cap or acrosome slipping off during massive expansion of the sperm head as based on their composition- the sperm head and head cap having different expansion rates.

The Skim Milk Extender had the lowest percentage abnormality (9.83%) which was slightly lower and similar to that obtained from the Zobo Extender (11.21%). The ability of the extension media to reduce the effects of cold shock as well as its ability to extract water from the spermatozoa might have accounted for this. Although when comparing cryopreservation to preservation at refrigeration and room temperature, significant differences were observed in all but the Skim Milk Extender. This showed that the type of extender used in cryopreservation might have an effect on the morphology of spermatozoa. This does not however tally with the works of Crusco dos Santos *et al.* (1999) who noted that cryopreservation affects the morphology of canine spermatozoa irrespective of the extender. However, her results might be due to the method of cryopreservation as this work employed the use of gradual freezing and thawing in order to reduce stress imposed on the spermatozoa.

4.4.5 ACROSOME INTEGRITY

Post thaw analysis of the cryopreserved spermatozoa revealed that Zobo Extender had the highest number of denatured acrosomes (8.54%) while the Skim Milk Extender had the least number of denatured acrosomes (Table 4.3). These results were not in line with the theory of expansion and contraction of the spermatozoa as the Zobo Extender should have been able to prevent the damaging effect of cryopreservation on the acrosome as a result of its composition. The high results might have been associated with the high mass activity associated with the Zobo

Extender (Table 4.2). High mass activity and motility increase sperm capacitation. Sperm capacitation results to acrosome reaction (Yanagimachi, 1994) which thus reduces acrosome integrity (Kommisrud *et al.*, 2002). An increase in mass activity would result in more friction between the progressively motile spermatozoa (the acrosome being the first contact in this case) as well as the spermatozoa coming in contact with one another. This is in line with Spindler *et al.* (2004) who observed that damaged acrosomes could also result from contact of spermatozoa with one another or even the plastic incubation tube. In addition to the above, the increase in mass activity will result in the removal of the outer glycoprotein coat of the spermatozoa, a criteria for spermatozoa to undergo capacitation. This may be observed in male spermatozoa which swim faster than their female counterparts but die faster (Donelson, 1998; Sircar, 2008).

The Sodium Citrate Glycine Extender which had 4.8% denatured acrosome (Table 4.3), showed that the effects of expansion and contraction of spermatozoa may not have had as much effects on the acrosome integrity as mass activity would. In the case of acrosome integrity, rapid expansion and contraction would have resulted in the slacking of the acrosome which acts as a head cap (due to the head expanding and contracting) and hence would slip off during extreme rapid expansion and contraction. Although the above results indicate that cryopreservation has an effect on the acrosome stability, the addition of Orvus ES Paste (OEP) as done by Tsutsui *et al.* (2000), might be an effective way of reducing this effect of cryopreservation on acrosome integrity.

4.4.6 DNA INTEGRITY

SCGE had the highest number of denatured DNA (Table 4.3, Fig. 4.6). This was followed by CWE (1.79%) and then Zobo and Skim Milk Extenders (1.67%). Compromisation of the DNA could affect the fertilizing ability of spermatozoa, Lewis and Aitkens (2005). The results showed that there is a 23% chance of the DNA to be denatures as opposed to a 77% chance of having a compromised acrosome during cryopreservation (Fig 4.7). Since the dye used only differentiates between the double stranded and single stranded DNA by colour emissions, this might not necessary mean the DNA content is lost but might only affect the pairing of Chromosomes. However, this work only looked into the splitting of the double stranded chromosomes as this affect spermatozoa fertility (Saikhun *et al.*, 2007). This is in line with the works of AJPH (1996) who reported that the DNA of an organism is the stable component of the organism containing

the personal information of the individual concerned and can be stored for long periods of years and even be used as a tool to tell the individuals future. The results on the DNA also tally with works of Donnelli *et al.* (2001) who observed no significant effects of cryopreservation on semen DNA. The results however differ from those of Kim *et al.* (2010) who observed that cryopreservation affected the DNA integrity of spermatozoa. Perhaps the method of freezing (rapid freezing/thawing or gradual freezing/thawing) might have made the difference in the results obtained as Szász, (2000) observed that in cryopreservation of canine semen, extenders had less influence on the post-thawing sperm quality than did the freezing rates.

4.5 CONCLUSION

From the experiment is can be deduced that artificial insemination using cryopreserved spermatozoa should be performed immediately after thawing as this is when progressive motility is highest. However, insemination using thawed semen can still be effective if insemination is done within the first 2hrs of thawing using Coconut Water Extender, Zobo Extender or Skim Milk Extender but not Sodium citrate Glycine Extender.

Although results between the Coconut Water, Skim milk and Zobo extenders were not significant, figures revealed that for inseminations performed after 3hrs post thaw, Zobo Extender would be the extender of choice as it maintained the spermatozoa above 50% as well as had the highest progressive motility after 3hrs.

Skim Milk extender was found on the average to be the mildest on spermatozoa as it reduced the effects of cold shock on spermatozoa, produced the highest progressive motility and also had its initial mass activity within the recommended range (Otite, 2000).

The experiment also revealed that Coconut Water and Zobo Extenders could still be used as their results were also in line with the accepted values for semen quality (Lofstedt, 2004; Otite, 2000; Günzel-Apel, 1994).

Since the DNA is less likely to be compromised during cryopreservation, it follows that in situations of zero percent motility where Intra Cytoplasmic Sperm Injection (ICSI) is to be

employed, cryopreserved semen using skim milk will still be effective as this method of cryopreservation results in the longest shelf life of the spermatozoa.

CHAPTER FIVE

EXPERIMENT 3

THE EFFECTS OF CHLOROQUINE PHOSPHATE ON CRYOPRESERVED CANINE SPERMATOZOA

5.1 INTRODUCTION

Although cryopreservation is the most appraised method of preserving canine semen over a long period of time or for use in cross border (long distance) breeding, cryopreservation has been observed to reduce fertility due to death of spermatozoa coupled with the low motility associated with the freezing and thawing process.

Even though this has been partially resolved through proper ovulation timing in the bitch as well as proper semen deposition, the results are still not as impressive as those obtained from insemination using fresh or chilled semen. One major attribute of this is the motility and progressive motility which is significantly reduced by cryopreservation.

There exists a need for thorough research to be conducted into revitalizing post thawed canine spermatozoa prior to insemination. Several methods abound on the use of additives for prolonging or revitalizing spermatozoa. Examples of such additives include, Ascorbic acid in bull semen (Kumar, 2002), chlorpromazine and catalase in bulls (Paudel *et al.*, 2010), Chloroquine phosphate as used in porcine semen (Egbunike, 1989) and bull semen (Kumar, 2002) and caffeine as demonstrated by Satish (2007). However, little information exists on the use of the above with regards to canine. In addition, research on this aspect in Nigeria is still elementary as the canine industry only kicked off a few decades ago as compared to the first world which can boast of a fully matured canine industry.

Cryopreserved semen has a reduced lifespan and hence calls for accurate timing of insemination. Missing the timing of insemination by as little as one day could result in zero conception. Due to the low motility and mass activity of the spermatozoa, breeders are able to achieve conception solely through intra uterine and surgical insemination methods. The later being more assured even though Fukushima *et al*. (2010) observed both guarantee the same results.

Since spermatozoa from fresh canine semen can take as short as 30min to an hr to reach the ova, boosting the mass activity of the post thawed canine spermatozoa could propel them to match up with the speed of fresh unextended semen. Such an increase in mass activity would mean that the speed of post thawed spermatozoa in the crawling state would be enhanced and hence this would result in an increase in progressive motility of the spermatozoa which would be visible under the light microscope.

The use of Chloroquine phosphate to boost sperm motility has been widely acclaimed in numerous animal species. In boars, Egbunike (1989) observed that Chloroquine was able to restore motility back to normal levels when motility drops due to prolonged storage. Kumar (2002) noted that Chloroquine diphosphate which acts as a membrane stabilizer improved the post-thaw quality of frozen semen of Jersey bulls.

This work investigates the use of Chloroquine Phosphate as a means of revitalizing post thawed canine spermatozoa.

5.2 MATERIALS AND METHODS

5.2.1 Experimental dogs: This was as described in section 3.2 (page 58).

5.2.2 Semen Collection: Semen samples were collected using the artificial balloon vagina (Otite and Egbunike, 2000) as described under section 3.3 (page 57).

5.2.3 Semen evaluation

Semen was evaluated for volume, concentration, mass activity, progressive motility, morphology, pH, acrosome integrity, and DNA as described in section 3.4 (pages 57-60).

5.2.4 Semen Extenders

Portions of the collected semen samples were preserved using 4 different extenders namely:

(i) Sodium-Citrate-Glycine Buffer and Extender (SCGE).

(ii) Coconut Water Extender (CWE).

(iii) Skim milk Extender (SME).

(iv) Zobo Extender (ZE).

5.2.5 Semen Extension

The collected semen was warmed alongside the prepared extender to body temperature (37^{o}C) and both were mixed together by gently inverting the mixture. Extension was done at a rate of 100 million spermatozoa to 1ml of extender.

5.2.6 Cryopreservation

Freezing of the extended semen was carried out using the process described in section 4.2.7 (page 85).

5.2.7 Thawing

Thawing was done at 37^{o}C as described in section 4.2.8 (page 85).

5.2.6 Post preservation analysis

All the frozen samples were later thawed prior to analysis. Analysis included mass activity, motility, concentration, pH, live/dead ratio and acrosome integrity.

5.2.7 Addition of Chloroquine Phosphate

Chloroquine Phosphate was added to portions of the extended semen (0.2ml of 3mM). This low rate of inclusion was chosen as chloroquine has been known to induce motility of spermatozoa in other species if added in lower concentrations (Hargreaves *et al.*, 1998).

5.2.8 Addition of Penicillin and Streptomycin

Penicillin and streptomycin were also added to all the extenders at a dosage of 10^5 i.u./1000ml and 100.00mg/100ml respectively as per Otite (2000).

5.2.9 Statistical analysis

The results obtained where compared using the analysis of variance ($p<0.05$) and their means subjected to Duncan's Multiple Range Test where applicable using SAS (2008).

5.3 RESULTS

With the addition of Chloroquine phosphate to the post thawed extenders, Skim Milk Extender (SME) had the highest initial progressive motility (Table 5.1). This was followed by Zobo Extender (ZE), Coconut Water Extender (CWE) and Sodium Citrate Glycine Extender (SCGE). The results obtained from the SCGE were significantly different from all other three extenders. There were no significance differences between the results obtained for the SME, CWE and the ZE. By the fourth hour, even though SME had progressive motility of 1%. These results were however not significantly higher than all other three extenders.

Chloroquine phosphate significantly affected the progressive motility of spermatozoa extended in SCGE and CWE (Table 5.2). Results obtained showed a negative influence of chloroquine phosphate in SCGE as the values for progressive motility were significantly lower upon inclusion of chloroquine phosphate while in CWE Chloroquine phosphate had a positive influence on the progressive motility of spermatozoa. Although the results obtained for ZE was higher, no statistical differences existed between their means. Upon an hour post addition of Zobo, the progressive motility had risen in all extenders (except SCGE). The higher figures obtained where however not significant in SME.

The initial Mass activity was significantly highest in SME and lowest in SCGE. Results obtained for ZE were not significantly different from SME and CWE though CWE and SME differed significantly (Table 5.3). By hour one, even though Zobo extender had the highest Mass Activity, these results did not differ significantly from CWE, SME and ZE.

Chloroquine Phosphate had significant effects on the mass activity of the extenders except ZE. These figures were higher in CWE, SME and ZE but not SCGE which had significantly lower values for MA.

The survival time of post thawed spermatozoa was significantly affected (negatively) by the addition of Chloroquine Phosphate. In all extenders, Chloroquine Phosphate reduced the survival time in all extenders with SME having the highest survival time of 5.83hrs (Table 5.5). This was followed by ZE (4.72hrs) CWE (4.78 hrs) and SCGE (2.77hrs).

Chloroquine had a significant negative effect on the percentage abnormalities in SME and ZE but not in SCGE and CWE. The percentage abnormality was highest in SCGE (18.46).

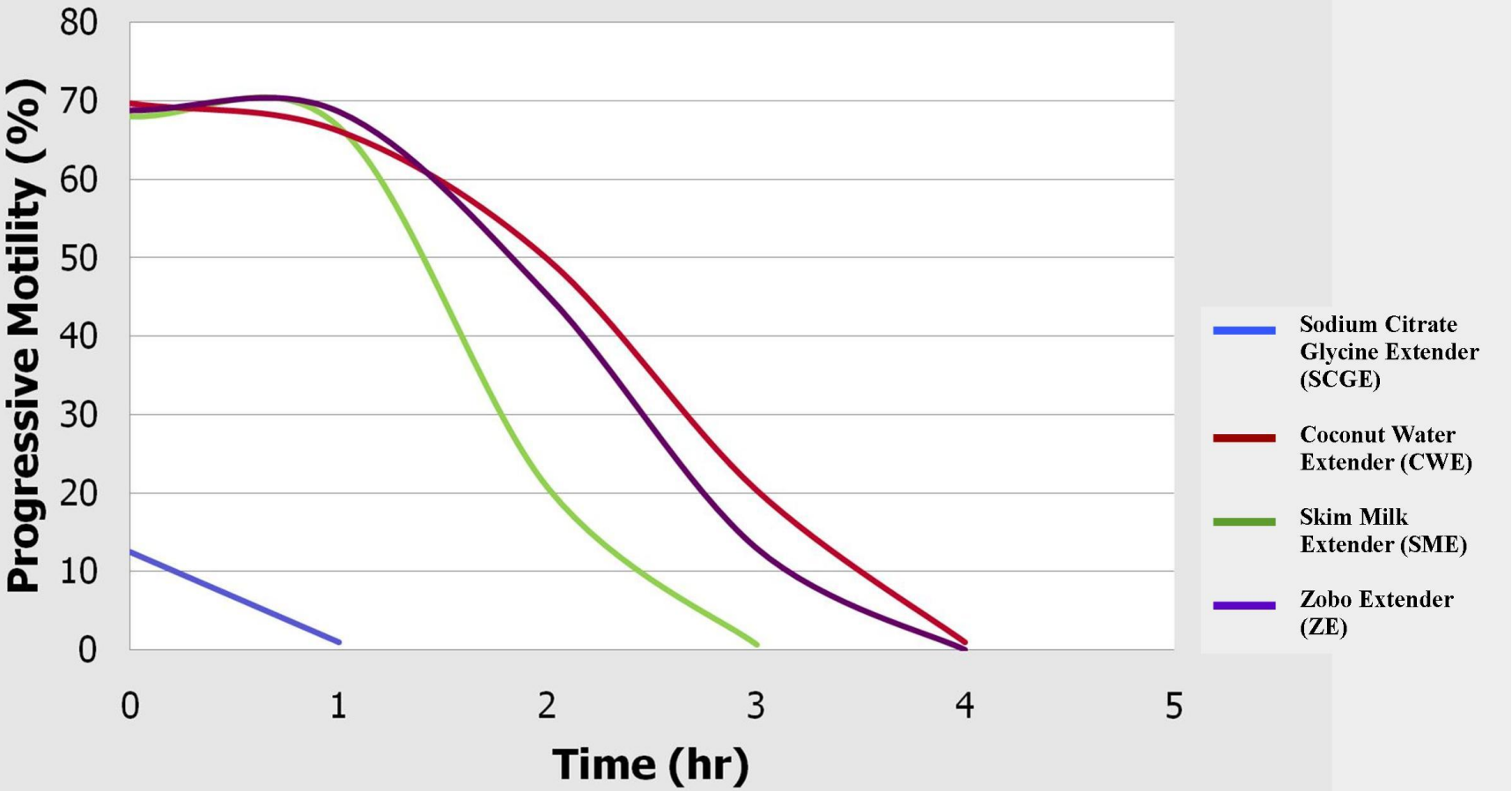


Fig 5.1: Effects of chloroquine on the progressive motility of cryopreserved canine spermatozoa

Table 5.1: The effects of Chloroquine Phosphate on the Progressive Motility of post thawed spermatozoa in 4 different extenders.

TIME (Hr)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
0	12.50^{b}	68.00^{a}	69.67^{a}	68.83^{a}	0.81
1	1.00^{c}	66.67^{ab}	66.17^{b}	68.67^{a}	0.25
2	0.00^{c}	20.67^{b}	49.83^{a}	45.25^{a}	0.98
3	0.00^{b}	0.67^{b}	20.41^{a}	13.00^{a}	1.32
4	0.00^{a}	0.00^{a}	1.00^{a}	0.00^{a}	0.12

Means on the same row with the different superscripts are significantly different ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

Table 5.2: The influence of chloroquine phosphate on the progressive motility of spermatozoa

Time (Hr)	Extender											
	SCGE			CWE			SME			ZE		
	NC	C	SEM	NC	C	SEM	NC	C	SEM	NC	C	SEM
1	30.92^{a}	12.50^{b}	1.88	65.25^{b}	68.00^{a}	0.22	71.17^{a}	69.67^{a}	0.37	68.83^{a}	68.83^{a}	0.31
2	13.00^{a}	1.00^{b}	1.55	61.50^{b}	66.67^{a}	0.26	65.83^{a}	66.16^{a}	0.21	63.83^{b}	68.67^{a}	0.39
3	3.67^{a}	0.00^{a}	0.56	50.17^{a}	20.67^{b}	0.67	49.67^{a}	49.83^{a}	0.64	53.25^{a}	45.25^{a}	1.43
4				37.75^{a}	0.67^{b}	0.86	46.92^{a}	20.42^{b}	0.99	47.08^{a}	13.00^{b}	1.61
5				16.25^{a}	0.00^{b}	0.68	23.00^{a}	1.00^{b}	0.61	19.83^{a}	0.00^{b}	0.61
6							6.17^{a}	0.00^{b}	0.51			

Means with different superscripts by extender on each row are significantly difference ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
NC: No Chloroquine
C: Chloroquine

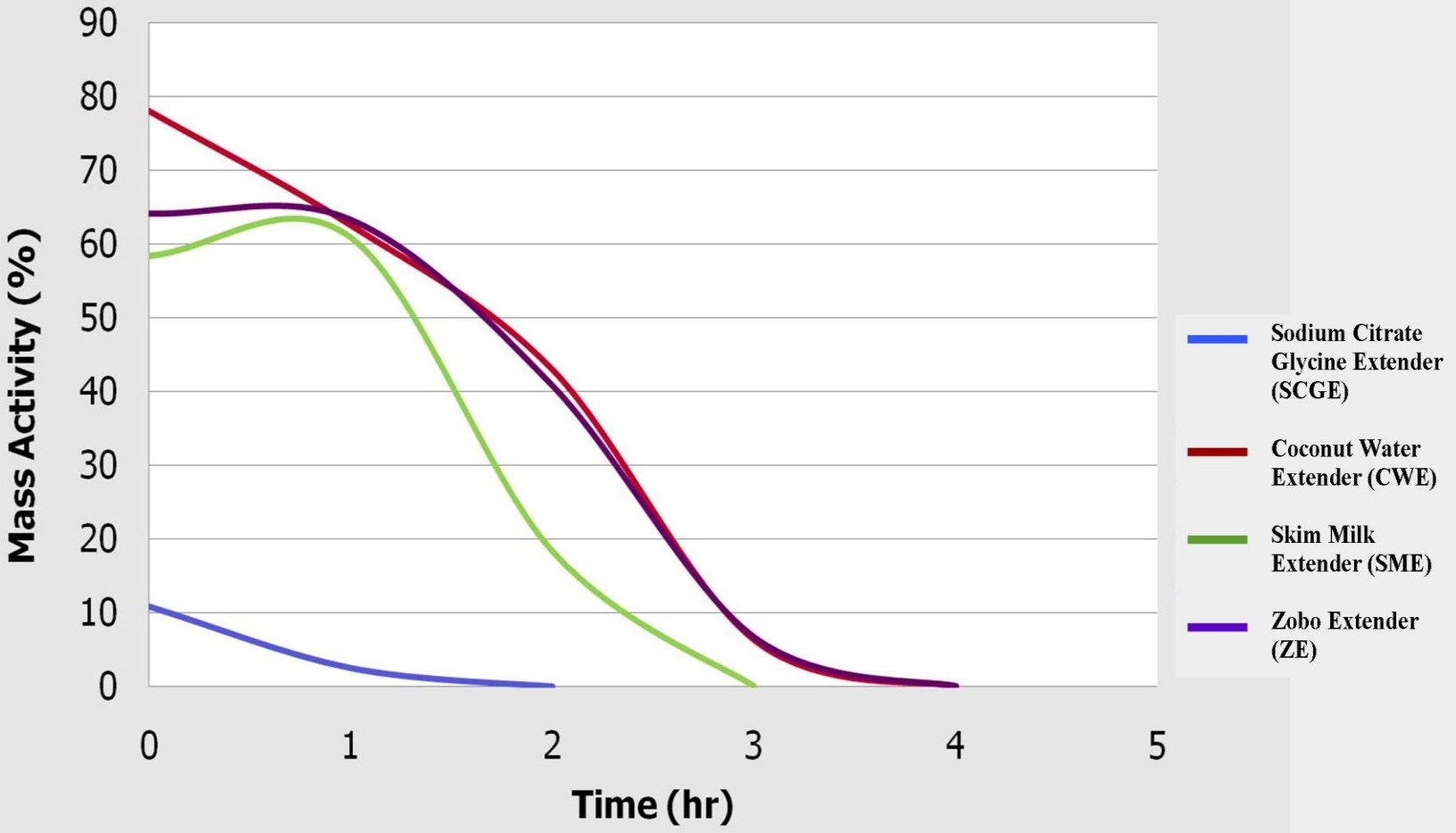


Fig 5.2: Effects of chloroquine on the mass activity of cryopreserved canine spermatozoa

Table 5.3: The effects of Chloroquine Phosphate on the Mass Activity of post thawed spermatozoa in 4 different extenders.

TIME (Hr)	EXTENDER				SEM
	SCGE	CWE	SME	ZE	
0	10.83^{c}	58.33^{b}	66.67^{a}	64.17^{ab}	0.98
1	2.50^{b}	60.83^{a}	62.50^{a}	63.33^{a}	0.86
2	0.00^{c}	18.33^{b}	42.92^{a}	40.83^{a}	1.71
3	0.00^{b}	0.04^{b}	6.25^{a}	6.67^{a}	0.77

Means with the different superscripts are significantly different ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
ZE: Zobo Extender

Table 5.4: The influence of chloroquine phosphate on the mass activity of spermatozoa

TIME (Hr)	EXTENDER											
	SCGE			CWE			SME			ZE		
	NC	C	SEM	NC	C	SEM	NC	C	SEM	NC	C	SEM
1	26.25^{a}	10.83^{b}	1.59	46.67^{b}	58.33^{a}	0.94	59.17^{b}	66.67^{a}	0.92	60.00^{a}	64.17^{a}	0.87
2	13.00^{a}	2.50^{b}	1.14	42.50^{b}	60.83^{a}	1.30	54.17^{a}	62.50^{a}	1.49	60.83^{a}	63.33^{a}	0.92
3	2.50^{a}	0.00^{a}	0.59	33.33^{a}	18.33^{b}	1.06	45.00^{a}	42.92^{a}	2.37	50.00^{a}	40.83^{b}	1.04
4				23.33^{a}	0.04^{b}	0.13	29.58^{a}	6.25^{b}	1.19	35.83^{a}	6.67^{b}	1.47
5				8.33^{a}	0.00^{a}	0.64	10.83^{a}	0.00^{b}	0.44	9.17^{a}	0.00^{b}	0.69
6							1.25^{a}	0.00^{b}	0.16			

Means with different superscripts by extender on each row are significantly difference ($p<0.05$)
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
NC: No Chloroquine
C: Chloroquine

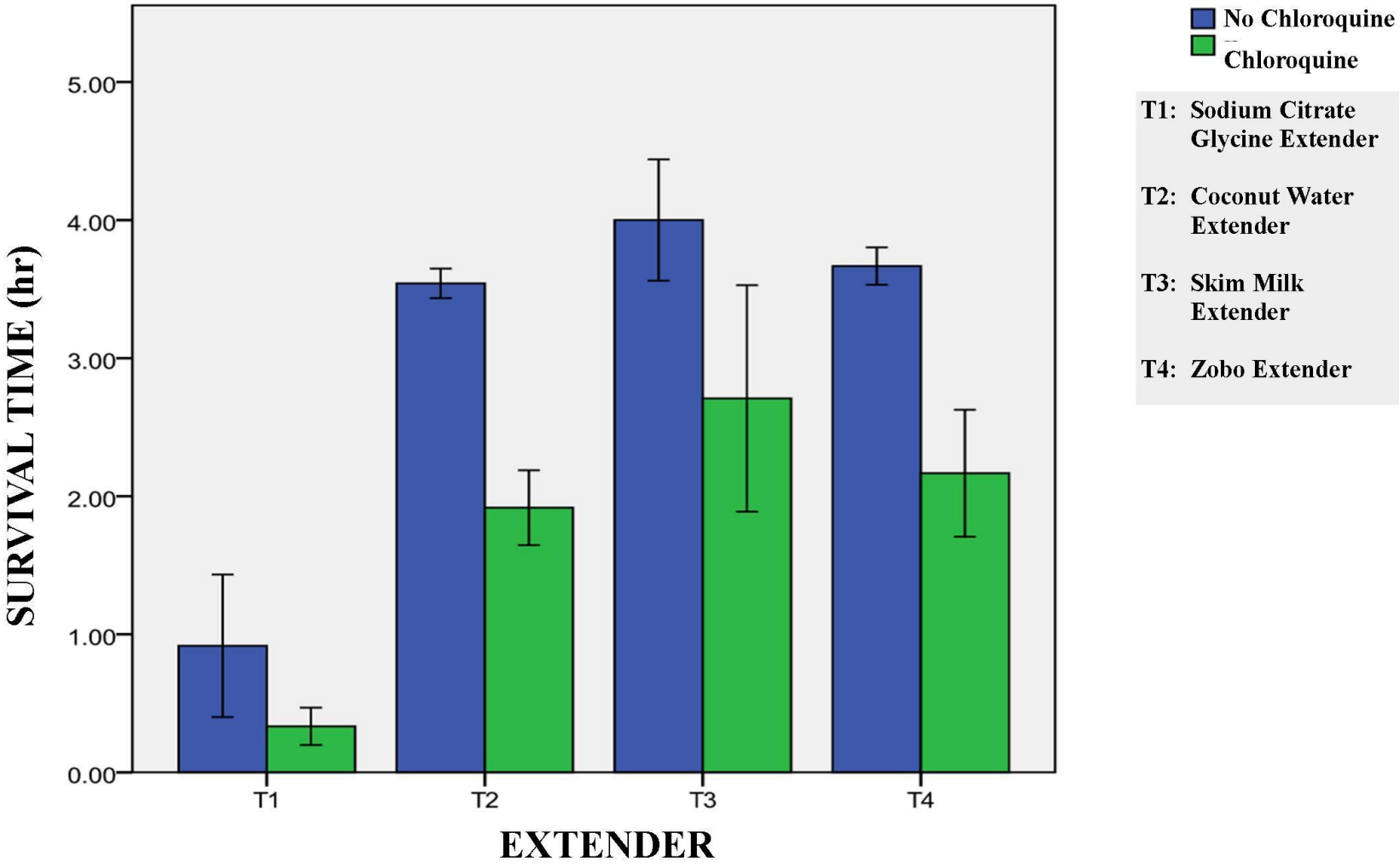


Fig. 5.3: Effects of Chloroquine Phosphate on the survival time of spermatozoa

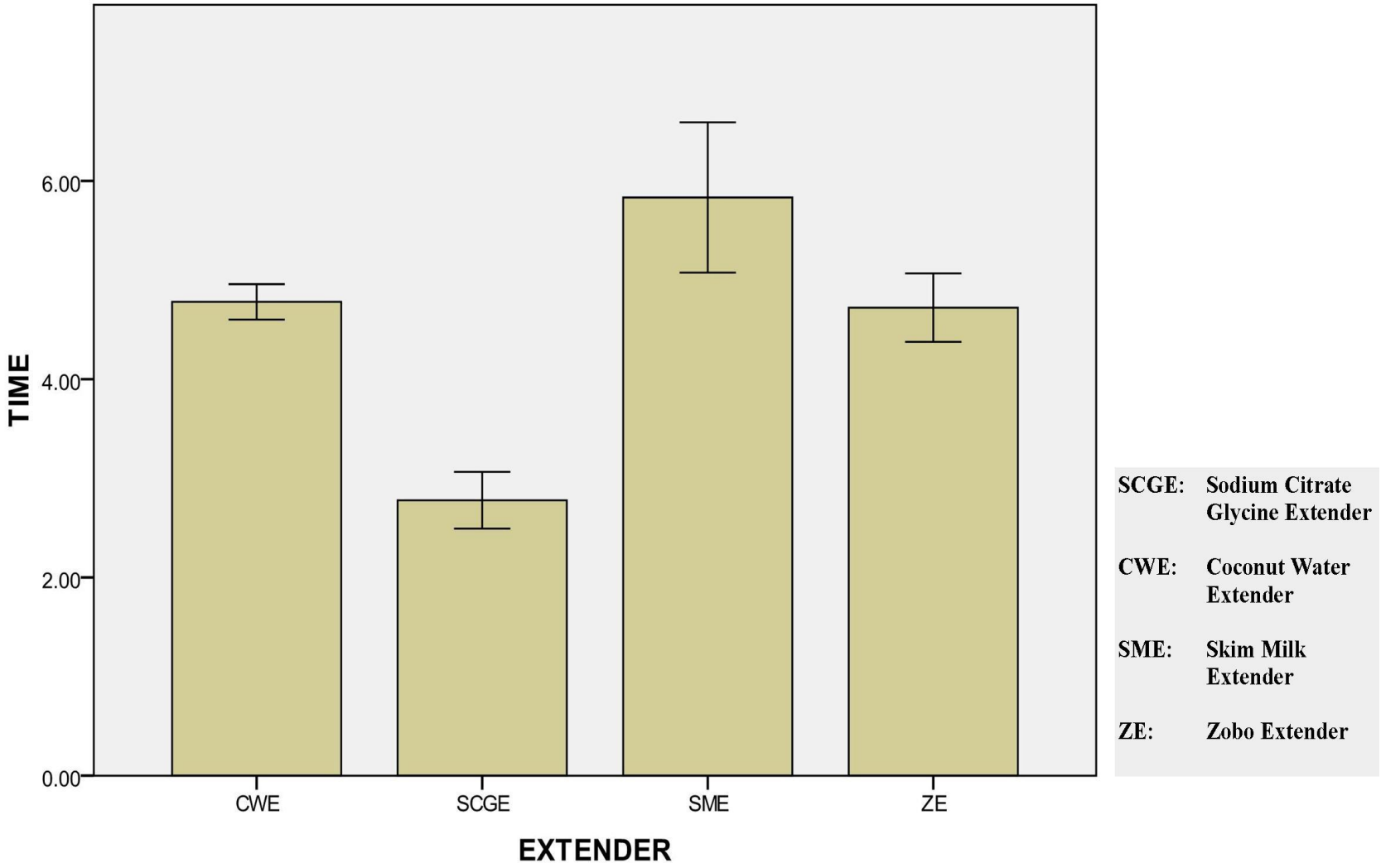


Fig. 5.4: Average survival time (hr) of spermatozoa with Chloroquine Phosphate

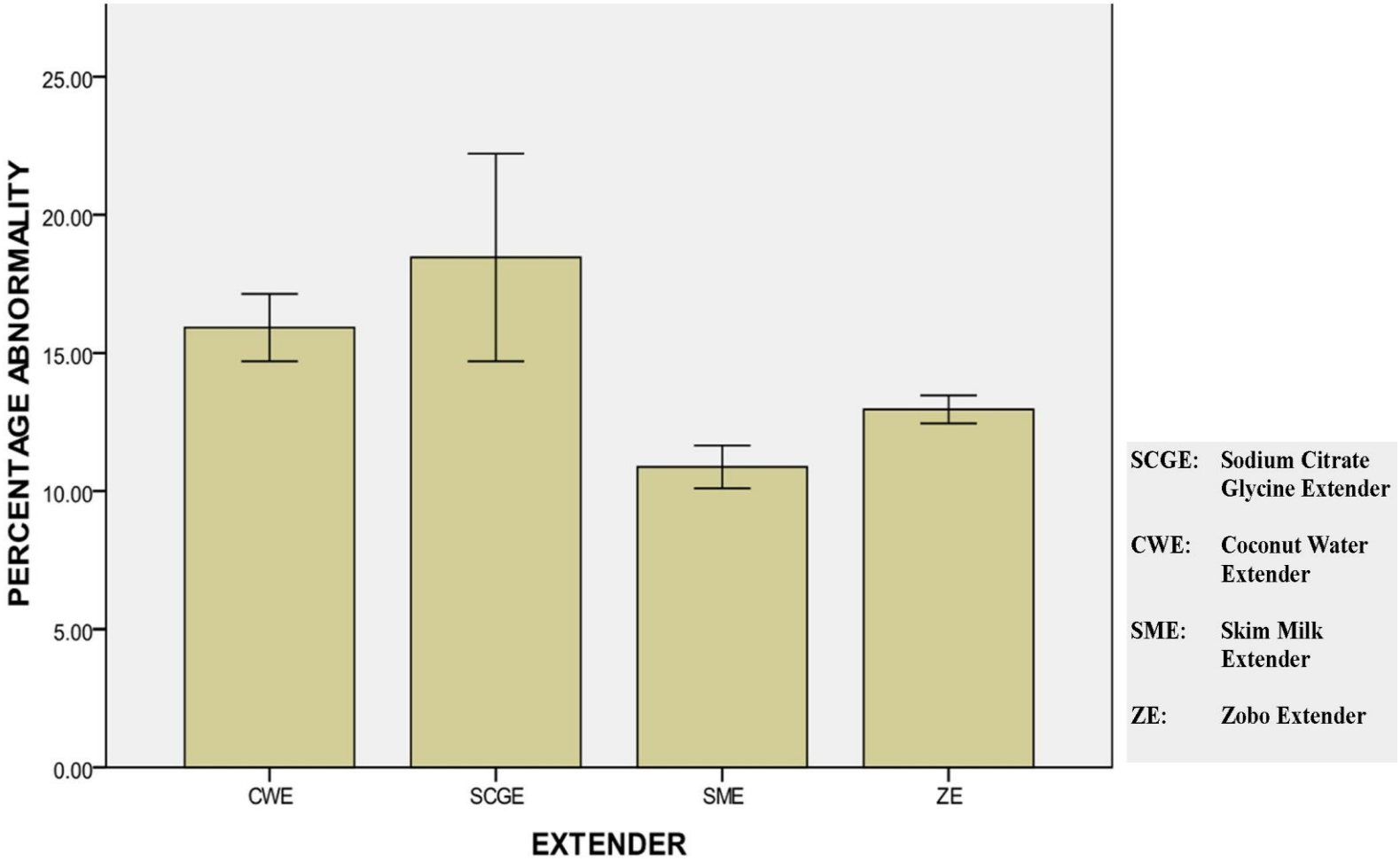


Fig. 5.5: Average percentage abnormality of spermatozoa with Chloroquine Phosphate

Table 5.5: Influence of Chloroquine Phosphate on the percentage abnormality and post thaw survival time of spermatozoa

PARAMETERS	EXTENDER											
	SME			ZE			SCGE			CWE		
	NC	C	SEM	NC	C	SEM	NC	C	SEM	NC	C	SEM
% Abnormality	9.83[b]	10.88[a]	0.27	11.21[b]	12.96[a]	0.26	15.29[a]	18.46[a]	1.14	13.67[a]	15.92[a]	0.72
Post Thaw survival time (hr)	7.67[a]	5.83[b]	0.07	5.83[a]	4.72[b]	0.08	3.67[a]	2.77[b]	0.06	5.61[a]	4.78[b]	0.07

Note: abc: Means on the same row with different superscripts differ significantly (P<0.05).
SEM: Standard Error of Mean
SCGE: Sodium Citrate Glycine Extender
CWE: Coconut Water Extender
SME: Skim Milk Extender
NC: No Chloroquine
C: Chloroquine

DISCUSSION

5.4.1 PROGRESSIVE MOTILITY

With the exclusion of Skim Milk and Zobo Extender, the results revealed that Chloroquine Phosphate has significant effect on the progressive motility of canine spermatozoa (Table 5.2). In most cases (excluding Sodium Citrate Glycine Extender), the effects of Chloroquine was not very glaring in the first hour with Chloroquine Phosphate either slightly increasing or decreasing the progressive motility.

The total effects of Chloroquine Phosphate (Table 5.1) on the initial post thaw progressive motility, showed no significant differences between the means of spermatozoa cryopreserved in Coconut Water, Skim Milk and Zobo Extenders. The three extenders had their progressive motility within the recommended range of 60% (Lofstedt, 2004).

Chloroquine Phosphate in Sodium Citrate Glycine Extender, was significantly detrimental to the progressive motility of spermatozoa (Table 5.2). Chloroquine also dropped the average progressive motility of the spermatozoa at a faster rate in this extender. By the second hour zero percent progressive motility was observed. The results implied that Sodium citrate Glycine Extender is not an ideal extender for preserving canine semen as the poor results obtained using this extender could not be revived by Chloroquine phosphate hence, leading to a waste in collection and preservation time culminating in economic loss.

The effects of Chloroquine Phosphate on spermatozoa cryopreserved in Coconut Water Extender was significant. Results showed that the addition of Chloroquine Phosphate significantly increased the progressive motility of spermatozoa extended in this extender. These results were significantly higher during the first two hours post thaw. These results tallied with other works done on various mammalian species (Egbunike, 1989) (Norman and Gombe, 1974) where they found that Chloroquine Phosphate significantly improved semen quality; hence the fertilizing potential of extended semen. The results also showed that while Chloroquine Phosphate increases progressive motility, it reduces the time taken for zero motility to be observed in cryopreserved spermatozoa. This is however of less significance as the life span of semen is extended in the reproductive tract of the bitch.

The effects of Chloroquine Phosphate on the progressive motility of spermatozoa extended in Skim Milk and Zobo Extenders were not higher during the first hour of addition of Chloroquine Phosphate but later increased during the second hour (significantly in Zobo Extender). It thus took a longer time for Chloroquine Phosphate to act on the spermatozoa. Skim Milk and Zobo Extenders both had the highest post thaw motility and mass activity; hence the slower response might have been due to the relationship between ATP and energy production as well as their relationship with spermatozoal mass activity.

5.4.2 MASS ACTIVITY

Although the results obtained with the inclusion of Chloroquine Phosphate were significantly different, all extenders (excluding Sodium Citrate Glycine Extender) had their mass activity above the minimum requirement of 50% (Otite, 2000) with the Zobo Extender having the highest mass activity or 66.67% (Table 5.3). This was followed by Skim Milk and Coconut Water Extenders respectively. It was also observed that the inclusion of Chloroquine Phosphate did nothing to revive the already weakened spermatozoa extended in Sodium Citrate Glycine Extender. Chloroquine however increased the mass activity of the spermatozoa extended in Coconut Water, Skim Milk and Zobo Extenders. These results were in line with those of Egbunike (1989) and Kumar, (2002) who observed that Chloroquine was able to revive quality of semen almost up to motility observed in fresh semen.

The effects of Chloroquine Phosphate on Mass activity, results showed that even though Chloroquine Phosphate raised the percentage mass activity, it was unable to sustain this increased mass activity for long. Hence, Chloroquine could increase fertilization in canines only when proper timing of oestrus and ovulation are used to determine the optimum time for insemination. This high mass activity would give spermatozoa the necessary push to penetrate the zona pellucida of the oocyte. Both hyperactivity and capacitation must go together for fertilization to occur (Suarez and Pacey, 2005). In addition, when semen is inseminated, there follows massive wave contractions which push the semen forward. However, the spermatozoa get attracted to the endosalpingeal epithelium and hence; an increased mass activity would enable them continue in the direction of the oocyte. This high activity would enable the spermatozoa to progress towards the oocyte swimming through the viscous substances such as the mucus and penetrate the oocyte wall. In areas with pockets of mucosa, hyperactivated sperm

are able to turn around (Suarez *et al.*, 1983). Hence, sperm can switch from swimming straight to turning around. In order for the sperm to penetrate the oocyte wall (ie the zona pellucida) it must undergo capacitation. This increased mass activity associated with chloroquine phosphate will increase capacitation (Suarez and Pacey, 2005).

This attribute of chloroquine phosphate to boost motility and mass activity might be brought about by cyclic AMP and would thus stimulate the respiration and motility of spermatozoa stored in vitro (Norman and Gombe, 1974) as studies have shown that stimulation of spermatozoal motility by cAMP is mediated by phosphorylations catalyzed by cAMP-dependent protein kinase (Tash and Means 1983). Thus, chloroquine phosphate may either be working as a catalyst or actually partaking in a phosphorylation reaction (Garbers *et al*., 1971a, b; Hoskins, 1973; Tash & Mann, 1973). Reducing the ratio of ATP to other adenine nucleotides results in an energy charge thus improving kinetic activity (displayed by flagella movement) and improving oxygen uptake (Whitehouse, 1967). Other researches have put it that chloroquine may either be working as a catalyst or actually partaking in a phosphorylation reaction (Garbers *et al*., 1971a, b; Hoskins, 1973; Tash & Mann, 1973). This work however supports the later as it showed that initially the spermatozoa used up the available ATP from its mitochondria (shown by the decline in motility) thus having an abundance of ADP (i.e. ATP $\rightleftharpoons$ ADP). Upon addition of chloroquine phosphate, it was acted upon thus transferring its phosphate to ADP, regenerating the ATP and then the spermatozoa resumed motility. As soon as the available ATP was used up, the spermatozoa lost motility again.

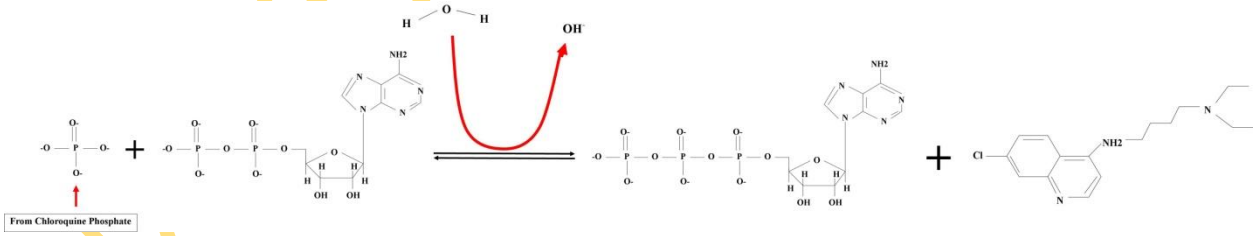


Fig. 5.6: Possible reaction showing the contribution of Chloroquine phosphate in the reaction converting ADP to ATP and vice versa.

This reaction could result in the production of an OH^- which in the presence of lactic acid may become H_2O_2 (hydrogen peroxide) or O_2 (oxygen). This production of OH^- affects the acidity of the medium which also explains the short life span of the spermatozoa.

One way of tackling this reduced life span as a result of acidity of the medium is to inseminate immediately after the inclusion of chloroquine phosphate as the bitches reproductive tract is alkaline in nature. This might explain the prolonged survival time of spermatozoa noticed in the bitch. With this an inseminator will be able to (a) increase the mass activity and motility of already weakened spermatozoa with chloroquine and (b) prolong the survival time of spermatozoa through an immediate insemination.

5.4.3 POST THAW SURVIVAL TIME

Although Chloroquine phosphate acts as a lysosomal stabilizer (Weissmann, 1969), and has some influence on the mass activity and progressive motility of spermatozoa, it reduced the overall post thaw survival time of spermatozoa (Table 5.5). These results are in line with the works of Carr *et al.* (1985) who observed a negative correlation between motility and survival time. Since rapid moving spermatozoa are more likely to undergo capacitation, their survival time is bound to be shortened. Norman *et al.* (1958) attributes this relationship to the gradual pH change of the medium resulting in an increase in lactic acid production. Please not that this build up in lactic acid is not all together detrimental in terms of Artificial insemination as in some instances, lowering the pH with lactic acid has been demonstrated to immobilize bull sperm (Acott and Carr, 1984; Carr *et al*., 1985). Immobilization is of benefit in sperm preservation. It might appear that there is a tolerant level for canine spermatozoa. Although the survival time appears short (ranging from 0.33hr in SCGE to 2.71hrs in SME, These results could create conception if ovulation and right time of insemination are timed and properly synchronized in bitches as spermatozoa have been recovered in the cranial reaches of the tubal ampulla only minutes after mating or insemination in humans (Settlage *et al*., 1973) and several other species of mammals (Overstreet and Cooper, 1978; Hawk, 1983, 1987).

These results also suggest that it would be erroneous to suggest that the opposing field of thought which states that spermatozoa do not thrive on the surrounding nutrients is right. The results of Chloroquine Phosphate's ability to provide the required phosphate needed to convert ADP to ATP which is necessary for energy production needed for flagella or tail movement testifies to this fact. The reduce post thaw survival time attributed to the high activity of the spermatozoa due to the inclusion of Chloroquine phosphate might be reduced by the inclusion of other

additives such as the addition of 0.5-1.0% OEP to the extender as this has been found effective in prolonging post-thaw survival time of canine spermatozoa (Tsutsu, 2000). In addition, this reduced life span might not be very viscible as oocytes are usually fertilized within hours of ovulation (Austin, 1957; Harper, 1994). In fact, Sperm can travel as far as the cranial reaches of the tubal ampulla within minutes of mating or insemination (Overstreet and Cooper, 1978; Hawk, 1983, 1987). Settlage *et al.* (1973) were able to recover spermatozoa at ovulation sites as early as 10 minutes after ovulation.

5.4.4 PERCENTAGE ABNORMALITY

Although the results obtained for the percentage abnormalities of spermatozoa in which Chloroquine Phosphate added when compared to the results obtained without Chloroquine Phosphate, were higher, these results only differed significantly in the Skim Milk and Zobo Extenders. Nevertheless, these results were within the accepted range for good quality semen.

The high progressive motility and mass activity associated with the addition of Chloroquine Phosphate might have been a major influence on the percentage abnormality of the extended semen. Results were however highest in Sodium Citrate Glycine Extender and lowest in Skim Milk Extender (Fig. 5.5)

5.5 CONCLUSION

The addition of Chloroquine Phosphate prior to insemination increases the quality of cryopreserved semen by rejuvenating the already weakened spermatozoa. Chloroquine Phosphate does not adversely affect the morphology of spermatozoa and hence its use in the canine industry as a boost to improving the fertilizing potential of cryopreserved semen is of vital importance in canine breeding. However, the increased mass activity associated with the addition of Chloroquine Phosphate does reduce the survival time of cryopreserved spermatozoa. Although it can be deduced from this experiment that Chloroquine can act as a booster to already weakened spermatozoa, this is largely dependent on the type of extender as its addition in the Sodium citrate Glycine Extender did not raise the quality of cryopreserved semen to recommended average required for effective artificial insemination in dogs. Although this is a pioneer work, it shows that Chloroquine Phosphate could be used to rejuvenate spermatozoa

extended in Coconut Water Extender and Skim Milk Extender while its inclusion rate in Zobo Extender still needs to be determined.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Semen preservation is of great importance in the canine industry. The choice of an extender for canine semen storage is largely dependent on the type and method of preservation (i.e. preservation temperature) as well as the length of storage prior to insemination.

If semen is to be stored at room temperature, Coconut Water Extender is the desired extender as inseminations performed hours after extension can still be used for artificial insemination. However, if semen is to be utilized immediately after extension, Zobo and Skim Milk Extenders are preferred as the figures obtained for their mass activity as well as initial progressive motility supersedes that of coconut water.

If semen is to be preserved in its chilled state, Zobo Extender should be used for preservation as its results superseded those of the other 3 extenders. This is of utmost importance if semen is to be preserved for as long as 9 days. However, if semen is to be used immediately within the first few days (not day one as there is no need for chilling if semen is to be used immediately), then any of the extenders should produce desirable results. However, the duration of survival of spermatozoa suspended in these extenders in the reproductive tract of the bitch was not studied in this experiment, then their effectiveness would largely depend on proper ovulation timing in the bitch.

Skim Milk Extender still remains the extender of choice for cryopreservation. Since inseminating immediately after thawing is recommended, then Coconut Water and Zobo Extenders can still be used as the values obtained still fall within acceptable range.

It has also been proven in this work that the addition of Chloroquine Phosphate prior to insemination does improve semen quality. Chloroquine Phosphate is also not hazardous to canine spermatozoa.

6.2 RECOMMENDATION

Although it is now proven that Chloroquine Phosphate improves semen quality, further work still needs to be done in this area on the exact amount of Chloroquine phosphate to be added in order to yield proper results.

The effects of Chloroquine Phosphate on the fertilizing ability of Canine Spermatozoa needs to be carried out through fertility tests using bitches in oestrus. In addition, additional additives such as OES might compliment the use of Chloroquine Phosphate and thus improve the quality of post thawed canine semen.

Since the first work to be done worldwide on the use of Zobo in extenders was done by Otite (2000), there is still a lot more to be discovered on the use of zobo as an extender for canine semen. A more effective zobo extender formula still needs to be discovered as zobo might thus be the replacement for other additives which are currently frowned at such as egg yolk and skim milk. Zobo might also be able to compliment other additives due to its buffering capacity.

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APPENDIX

COMPOSITIONS OF THE DIFFERENT SEMEN EXTENDERS

(i) SCGE

BUFFER	EXTENDER
Sodium citrate dehydrate 10.00g	Buffer 70 (ml/100ml)
Glycine 5.00g	Egg yolk 30 (ml/100ml)
Sulfanilamide 3.00g	Penicillin 10^5 (i.u./100ml)
Boiled distilled water 1000.00ml	Streptomycin 100.00 (mg/100ml)

(ii) CWE

BUFFER	EXTENDER
Sodium citrate dehydrate 28.00g	Coconut milk 15ml
	Egg yolk 25ml
	Streptomycin 135.00mg
	Penicillin 300 i.u

(iii) SME

BUFFER	EXTENDER
Sodium citrate dehydrate 15.30g	Buffer 80ml
Potassium citrate 0.40g	Skim milk 4.00 (ml/100ml)
Citric acid 1.30g	Penicillin 10^5 (i.u./100ml)
Glucose 15.00g	Stroptomycin 100 (mg/100ml)
Boiling distilled water 1000ml	Egg yolk 6.00 (ml/100ml)

(iv) ZE

BUFFER	EXTENDER
inseminations yielded a higher whelping rate ($P < 0.05$) and greater mean litter size ($P < 0.05$) than that of one insemination, 77% and 5.6 +/- Sodium citrate dehydrate 20.50g	Zobo 4.00 (ml/100ml)
Sodium bicarbonate 2.10g	Penicillin 10^5 (ml/100ml)
Potassium chloride 0.40g	Streptomycin 100 (ml/100ml)
Glucose 6.00g	Egg yolk 10 (ml/100ml)
Sulphanilamide 3.00g	
Boiled distilled water 1000ml	

PREPARATION OF BUFFERS

(i) Weigh ingredients of buffer into a 1000ml volumetric flask

(ii) Boil distilled water for 10 minutes and partially fill volumetric flask, rotating the flask in order to dissolve weighed ingredients thoroughly

(iii) Cool flask and add more boiled distilled water to bring the buffer up to the 1000ml mark on the volumetric flask

PREPARATION OF EXTENDERS

(i) Pour required amount of buffer into a 100ml graduated cylinder

(ii) Add additives (skim milk, zobo, coconut water etc)

(iii) Add required amount of egg yolk (note egg shell must be sterilized by cleaned with alcohol before separating yolk from albumen with a separator).

(iv) Stopper and shake thoroughly to mix buffer and additives

(v) Centrifuge mixture at 3000 rpm for 10 minutes

(vi) Decant supernatant

(vii) Add 0.desired amount of prepared solution of penicillin and streptomycin (or other desired antibiotics) to supernatant

** Ensure to use fresh extender only.

PREPARATION OF ADDITIVES

Zobo (*Hibiscus sabdarifa*)

(i) Weigh 2g of dried zobo leaves
(ii) Put leaves in a 250ml beaker
(iii) Fill beaker to the mark with distilled water
(iv) Boil for 15 minutes
(v) Filter and add to extender

** Tips for identifying the right concentration of zobo: Pour in zobo in drops (as in titration). Stop as soon as the colour of extender changes to light blue/purple (not red).

Coconut water (*Cocos nucifera*)

(i) Clean coconut shell to remove dust and other particles
(ii) Make small incision through shell
(iii) Pour liquid contents of coconut into a 250ml beaker
(iv) Boil for 15 minutes
(v) Filter and add to extender

Skim milk

(i) Pour skim milk into beaker
(ii) Bring to boil and leave boiling for 10 minutes
(iii) Add skim milk to extender