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EFFECT OF THE ACIDIC BUFFER 2-[N- MORPHOLINO] ETHANESULFONIC ACID ON FROZEN-THAWED BULL SEMEN

By

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Submitted in partial fulfillment of the requirements for the degree of *Magister Scientiae* (Veterinary Science) in the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria

PROMOTOR: PROF DAVID GERBER

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My Creator most of all, who has never failed me.

DEDICATION

This thesis is dedicated to my beloved parents

Nico and Ottelien Botha

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DECLARATION

I, **Alma Ester Botha,** hereby declare
that the work on which this thesis is based is original
and that neither the whole work or part of it
has been, is being or shall be submitted for another degree
at this or any other university, institution for tertiary education
or professional examining body.

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SUMMARY

EFFECT OF THE ACIDIC BUFFER 2-[N- MORPHOLINO] ETHANESULFONIC ACID ON FROZEN-THAWED BULL SEMEN

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Degree: MSc

The aim of the current study was to determine if frozen-thawed bull semen can be treated with the acidic buffer MES (2-[N- morpholino] ethanesulfonic acid) without any detrimental effect on the motility, plasma membrane, acrosomal membrane and longevity of sperm.

Frozen bull semen was obtained from a local co-operative. The semen was frozen in 0.25 mL French straws at a concentration of 80 x 10^6 sperm cells per millilitre. Semen of two different batches from ten bulls of four different breeds was used in this study.

Three frozen semen straws of each batch were thawed at 38° C for 25 seconds. The thawed semen was pooled and then split into two aliquots. The one aliquot was used as control, whilst the other was exposed to MES treatment. The motility, plasma membrane integrity, acrosomal membrane integrity and longevity of sperm were evaluated.

The effect of MES on motility was minimal as only the percentage of aberrantly motile sperm increased two hours after treatment. Although no effect on the plasma membranes were observed, it can be assumed that some damage did occur due to the fact that the acrosomal membranes were affected significantly. No significant effect was found for longevity of sperm between the control and treated samples, but a significant effect was found for both the control and treated samples over time.

Although the detrimental effects caused by MES treatment would render some sperm unable to fertilise an oocyte, it is likely that a sufficient portion of sperm would survive the treatment. It is probable that this treatment would also be effective in frozen-thawed buffalo semen. The following step would be to treat semen of foot-and-mouth disease positive bulls with MES to establish if treatment with MES will be effective in inactivating foot-and-mouth disease virus in semen of infected bulls.

SAMEVATTING

Die doel van hierdie studie was om te bepaal of bevrore-ontdooide bul semen met MES (2-[N- morpholino] ethanesulfoniese suur), ‘n suurbuffer, behandel kan word sonder enige nadelige effek op die beweeglikheid, plasmamembraan integriteit, akrosoommembraan integritieit en die langlewendheid van sperma.

Bevrore bul semen was van ’n lokale koöperatiewe maatskapy bekom. Die semen was in 0.25 mL Franse strooitjies bevries teen ‘n konsentrasie van 80 x 10^6 sperma per milliliter. Semen van twee verskillende lotte van 10 bulle van vier verskillende rasse was vir die studie gebruik.

Drie bevrore semen strooitjies van elke lot was by 38° C ontdooi vir 25 sekondes. Die ontdooide semen was saamgevoeg en toe in twee gelyke deelvolumes verdeel. Die een deelvolume het as die kontrole monster gedien terwyl die ander monster aan MES blootgestel was. Die beweeglikheid, plasmamembraan integriteit, integriteit van die akrosoommembraan en langlewendheid van sperma was geevalueer.

MES het ‘n minimale effek op die beweeglikheid van sperma gehad aangesien daar slegs ‘n verhooging van die persentasie abnormale bewegende sperma twee ure na die behandeling gevind was. Alhoewel geen effek van die behandeling op die integriteit van die plasmamembraan waargeneem was nie, kan aanvaar word dat die plasmamembraan wel beskadig was omdat die effek van die behandeling op die akrosoommembraan betekenisvol was. Vir langlewendheid van sperma was geen noemenswaardige effek gevind tussen die kontrole en die behandelde monsters nie,

maar ‘n noemenswaardige effek was gevind in langlewendheid van sperma van beide die kontrole en behanderde monsters oor ‘n periode van tyd.

Alhoewel die nadelige uitwerking wat MES behandeling op sommige van die sperma gehad het sal veroorsaak dat die sperma nie in staat sal wees om ‘n eiersel te bevrug nie, het ‘n noemenswaardige groot deel van die sperma waarskynlik die behandeling oorleef. Hierdie behandeling sal waarskynlik ook doeltreffend wees in bevrore-ontdooide buffel semen. Die volgende stap sal wees om semen van bek-en-klou- seer positiewe bulle met MES te behandel om te bepaal of die behandeling met MES in staat sal wees om die bek-en-klou-seervirus in die semen van bek-en-klou-seer positiewe bulle te inaktiveer.

LIST OF ABBREVIATIONS

abm	= Aberrantly motile
AI	= Artificial insemination
ATP	= Adenosine triphosphate
° C	= Degree Celsius
CASA	= Computer assisted sperm analysis
cAMP	= Cyclic adenosine monophosphate
Co	= Control sample
Fig	= Figure
FMD	= Foot-and-mouth disease
FMDV	= Foot-and-mouth disease virus
g	= Gram
imm	=Immotile
IVP	= *In vitro* produced
km	= Kilometer
KNP	= Kruger National Park
MES	= 2-[N-morpholino] ethanesulfonic acid
m	= Meter
mg	= Milligram
mL	= Milliliter
min	= Minutes
mm	= Millimeter
PBS	= Phosphate buffered saline
Prm	= Progressively motile

R S A	= Republic of South Africa
sec	= Second
TALP	= Tyrode’s Albumin-Lactate-Pyruvate
Tr	= Treated sample
µL	= Micro liter
ZAR	= South African rand

CHAPTER 1

INTRODUCTION

1.1 Background and motivation

1.1.1 Background

Foot-and-mouth disease (FMD) is one of the most contagious animal diseases of livestock in Southern Africa. Even though foot-and-mouth disease virus (FMDV) has no or only little effect on buffalo, the fact that buffalo are instrumental in maintaining FMDV has vast economic consequences for agriculture and especially game farming in Southern Africa (Thompson, 1996). The 2001 outbreak of FMD in the United Kingdom caused losses of more than 8 billion pounds. The total operational costs for the national Department of Agriculture to contain the FMD outbreak in the Republic of South Africa (RSA) from September 2000 to February 2001 are estimated at 90 million ZAR. FMD has been recognized as the most important restriction to the international trade in animals and animal products (Leforban & Gerbier, 2002). Mechanisms must be developed whereby buffalo can be made more easily available to game farmers and ecotourism without limiting agricultural developments (Thompson, 1996). Outbreaks of FMD have occurred in every livestock-containing territory in the world except in New Zealand (Pharo, 2002; Grubman & Baxt, 2004).

In the RSA, FMD is listed as a controlled animal disease in terms of the Animal Diseases Act, Act 35 of 1984. FMD is caused by a virus of the family *Picornaviridae*, genus *Aphthovirus*. Seven distinct immunological serotypes are known: A, O, C, SAT1, SAT2, SAT3 and ASIA1. Six of the seven serotypes (all except ASIA1) are

common in Africa. The three Southern African Territories (SAT) serotypes are unique to Africa. (Bastos, 1998; Vosloo *et al.,* 2002). In Southern Africa the African buffalo (*Syncerus caffer)* is the major maintenance host of SAT serotypes and is instrumental as a reservoir of the virus (Hedger, 1976; Bengis *et al.,* 1987). Because a carrier state exists in some animals, once an animal is infected, the infection is considered as lifelong. In cloven-hoofed livestock FMD is characterized by high morbidity and low mortality. Affected animals develop vesicles and erosions at multiple sites, usually in the mucosa of the mouth and on the skin of the interdigital areas and the coronary bands. These vesicles and erosions are generally preceded by fever (Thompson & Bastos, 2004).

In the RSA, the Kruger National Park (KNP) is regarded as a FMD infected zone. The rest of the RSA is recognized by the World Organization for Animal Health (OIE) as a FMD free zone (OIE, 2007). Measures to contain and manage FMD outbreaks in RSA are in place.

The KNP is situated in the north-eastern part of the RSA and is the FMD infected zone. The park is about 60 to 80 km wide and 350 km long and is fenced off by a 2.4 m electrified fence. Game inspections are performed on a regular basis and any suspected FMD cases are reported to the state veterinarian. In terms of the Animal Diseases Act of 1984, FMD is a controlled disease (National Department of Agriculture, 2008).

The FMD buffer zone consists of the area bordering the southern and western border of the KNP which is about 10 to 20 km wide and about 350 km long. All cattle in the buffer zone are vaccinated every 6 months and inspected weekly for FMD. Vaccinated cattle are branded with an ‘F’ on the right side of the neck (National Department of Agriculture, 2008).

The surveillance zone is situated to the south and the west of the buffer zone and is about 10 km wide and 350 km long. All cattle are inspected every 14 days. In this zone no FMD vaccination is permitted. The rest of the control zone is situated to the west of the surveillance zone as well as an area about 10 km wide along the national borders of RSA with Swaziland, Botswana, Zimbabwe and Mozambique. This is an extra protection zone and cattle are inspected every 28 days. The FMD control zones around the endemic areas to monitor a possible outbreak and to control the spread of FMD are shown in Figure 1.1 (National Department of Agriculture, 2008).

1.1.2 Motivation

Although African buffalo (*Syncerus caffer)* have maintained a high genetic diversity, in spite of historically known population bottlenecks, the African buffalo from the KNP are in general bigger and have a wider horn span than buffalo from other areas in South Africa. This increases their economic value from a conservational, trophy hunting and ecotourism viewpoint (Wenink, 1998).

Currently the only method to conserve and reproduce the genetics of the buffalo of the KNP and other FMD positive buffalo elsewhere, is through the utilization of costly breeding programmes. The cost of producing a disease-free buffalo amounts to ZAR 70 000 (Hunt, 2008). A number of registered breeding farms exist in RSA and Zimbabwe, where infected buffalo within the FMD control zones are used to breed FMD-free buffalo calves. Different protocols exist:

- calves remain with the buffalo cows until passive immunity to FMDV decline, usually at 12 months, and are then removed before they become infected; or
- calves are removed at birth and are not allowed to suckle and ingest colostrum and are subsequently allowed to be nursed by sero-negative dairy cows acting as foster mothers; or

calves are removed from their sero-positive mothers and are hand-raised. (Condy & Hedger, 1978; Vosloo *et al.,* 2002; Hunt, 2008).

In general these projects have been quite successful and a few hundred animals that were raised according to these protocols have been introduced to the FMD-free zones (Vosloo *et al.,* 2002).

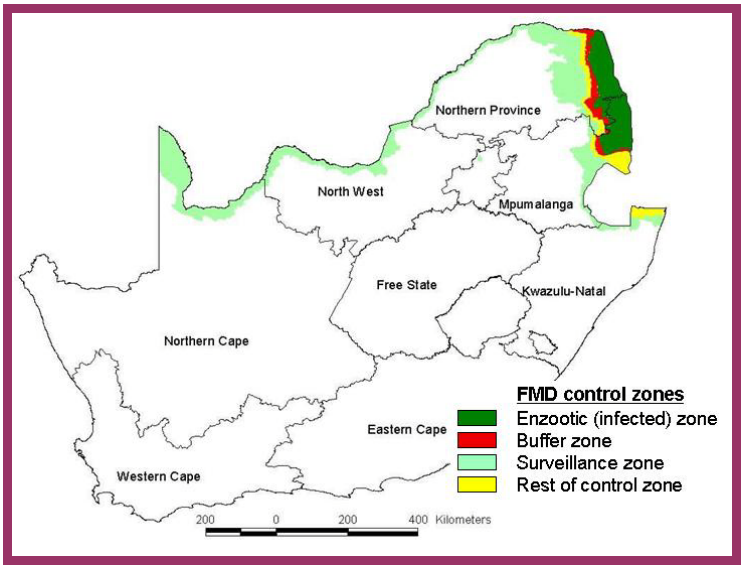

Figure 1.1: Foot-and-mouth disease (FMD) control zones in South Africa (National Department of Agriculture, 2008).

The FMDV can be preserved by refrigeration and freezing but is progressively inactivated by temperatures above 50º C. The FMDV is very sensitive to pH and is known to be inactivated highly efficiently by a pH <6.0 or > 9.0. The acidic physiological buffer 2-[N-morpholino] ethanesulfonic acid (MES), with a pH of 5 destroys FMDV within seconds due to the low pH (Acharya *et al.,* 1990; Thompson & Bastos, 2004).

Artificial insemination and assisted reproductive techniques may prove to be of vital importance to avoid the problems regarding the transport of African buffalo, whilst still promoting the genetic pool in the FMDV infected areas (Gerber, 2000). Artificial insemination (AI) was the first reproductive technology to be applied commercially for the genetic improvement of animals in the mid-1900s. The advantages of AI in terms of disease control are well recognized (Farin *et al.,* 2007). Cryopreservation of semen has been widely used around the world to preserve specific genetic traits over an extended period. The storage of semen under artificial conditions can be achieved by applying methods that decrease or arrest the metabolism of sperm and thereby prolong their fertile life. Handling, freezing and thawing of semen do however have a detrimental effect on some sperm characteristics such as motility and membrane stability (Salamon & Maxwell, 2000).

In studies performed on humans, the virus load of semen from men who tested positive for HIV-1 and Hepatitis C virus could be lowered to undetectable levels by washing and swim-up separation of the semen (Hanabusa *et al.,* 2000; Pasquier *et al*., 2000). Jooste (2004) found that *in vitro* produced (IVP) embryos exposed to FMDV can be rendered free of the virus and that embryos can be cleared of infective FMDV. The application of the method described by Jooste (2004) may enable the production of IVP embryos in FMD endemic countries and subsequent movement of these embryos to other countries.

Up to date no research has been done to determine the effect of MES on the viability of semen. The development of methods to decrease the risk of transmitting FMD via semen may possibly reduce the risk of transmitting the disease if treated semen proves to be free of the virus. With the application of assisted reproductive techniques the valuable gene pool from the buffalo of the KNP and FMD positive buffalo elsewhere could be conserved.

1.2 Research question

The aim of this study was to determine if frozen-thawed bull semen can be treated with the acidic physiological buffer MES without any detrimental effect to motility, longevity, acrosomal membrane integrity and plasma membrane integrity. Frozen-thawed bull semen was used as a model for African buffalo (*Syncerus caffer)* semen.

1.3 Hypotheses

Exposing frozen thawed bull semen to a MES buffered solution (pH 5) has no effect on its motility, longevity, acrosomal membrane integrity and plasma membrane viability.

1.4 Work plan

The work plan for this study was structured as follows:

- Review the literature on FMD in RSA and its effects on African buffalo (*Syncerus caffer)* and cattle.
- Review the literature on the anatomy and physiology of the bovine reproductive tract and semen as a model for the African buffalo (*Syncerus caffer).*
- Review the literature on:
    - the thawing of frozen bull semen;
    - staining technique using a vital stain;
    - evaluation of sperm motility;
    - plasma membrane integrity,
    - acrosome integrity; and
    - longevity of sperm.

- Collect frozen semen form several different breeds from Taurus Co-operative (Irene, South Africa, 0062) to ensure a standardized source.
- Thaw frozen semen and split the samples into control (Co) and treated (Tr) samples.
- Expose the Tr samples to MES.
- Evaluate the following variables of the Co and Tr semen samples:
    - motility over specified time periods;
    - plasma membrane integrity;
    - acrosome integrity; and
    - longevity over specified time periods.
- Perform statistical analysis of data obtained.

CHAPTER 2

LITERATURE REVIEW

2.1 Foot-and-mouth disease and semen

2.1.1 The African buffalo (*Syncerus caffer)*

Historically, African buffalo populated almost the entire sub-Saharan Africa. African buffalo were almost extinct during a rinderpest pandemic (morbillivirus) that affected the African continent at the end of the 19th century. Although many other wild ruminant populations were affected, buffalo suffered most; the mortality was estimated at between 90 and 95% (Plowright, 1982). In 1902 only 20 buffalo were reported to survive in the KNP and in 1929 only 75 were reported in the Umfolozi-Hluhluwe Complex (Stevenson-Hamilton, 1911; O'Ryan *et al.,* 1998). As a result of habitat fragmentation due to human habitation most of the African buffalo populations are currently limited to protected areas. Of the more than 3 million buffalo that wandered the African plains only some 400 000 remain (Lessard *et al.,* 1990).

At present the numbers of African buffalo are very favorable in the RSA and the species is currently not regarded as endangered. Nevertheless, the African buffalo belongs to Africa's "Big Five" and currently there is a big demand for "disease free" animals for trophy hunting and breeding purposes. The current demand for "disease free" buffalo exceeds the availability (Winterbach, 1998; Roux, 2008).

2.1.2 Structure of the foot-and-mouth disease virus

FMDV belongs to the family Picornaviridae, genus *Aphthovirus* and is a non-enveloped single stranded RNA virus with a spherical shape that displays 20-sided proportions. The outer coat of protein that surrounds the virus has a diameter of 27 – 28 ηm. The virion roughly consists of 70% protein and 30% RNA and a small amount of lipid. It has a molecular mass of about 8, 5 x 10^6 Dalton (Acharya *et al.,* 1989; Acharya *et al.,* 1990; Thompson & Bastos, 2004). Figure 2.1 shows an image of a FMD virion.

The FMDV can be preserved by refrigeration and freezing but is progressively inactivated by temperatures above 50º C. It is known to be inactivated by a pH <6.0 or > 9.0 and will undergo changes in even mildly acidic solutions (Thompson & Bastos, 2004; OIE, 2007).

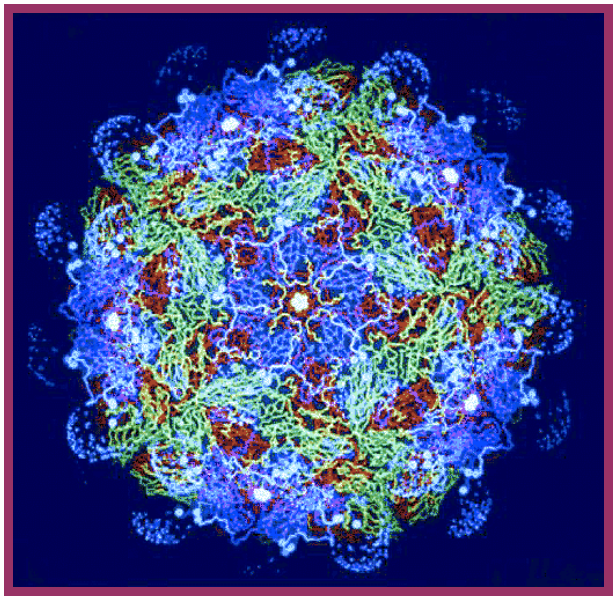

Figure 2.1: Image of a foot-and-mouth disease virion at 2.9 Å resolution showing the relative position of four structural proteins (blue, green, red and white) (Acharya, *et al.* 1990)

2.1.3 Transmission of foot-and-mouth disease

The FMDV rapidly replicates and spreads within the infected animal. For less than two weeks after being infected, quantifiable amounts of FMDV are present in excretions and secretions of affected animals. Differences between virus strains and host species do exist (Burrows, 1968; Hyde *et al.,* 1975; Donaldson, 1987; Kitching, 1992; Thompson & Bastos, 2004). In African buffalo and domestic ruminants the pharynx and dorsal soft palate is the site of preference for FMDV duplication. The virus can be present in the pharyngeal area for several years after the acute phase of infection (Hyslop, 1970; Graves *et al.,* 2001; Thompson & Bastos, 2004).

The nasal passages, oesophago-pharyngeal area and the urogenital tract also contain considerable amounts of virus and the virus can be shed up to four days prior to the manifestation of lesions (Thompson & Bastos, 2004).

Although FMD is a highly contagious disease not all the possible routes of transmission are entirely understood. Based on field trials performed in RSA and Zimbabwe it was found that venereal transmission of the disease is likely to occur during the acute phase of infection. It is not yet known if the virus can be recovered from semen after the acute infection phase. (Bastos *et al.,* 1999). Artificial insemination is thus also a potential avenue of viral transmission. Therefore all semen used in breeding programs has to be collected from bulls that are FMD-free and the absence of the virus in the semen has to be established (Thompson & Bastos, 2004). Currently little is known about the association of FMDV with semen.

If live FMDV can be isolated from an animal 28 days, or later after the infection, the animal is defined as a carrier (Sutmoller *et al.,* 1968). The carrier state in domestic

cattle can continue for as long as 3.5 years and in African buffalo for as long as 5 years. In wild African buffalo the carrier rate can be as high as 50 to 70% (Condy *et al.,* 1985; Alexandersen *et al.,* 2002).

FMD is a zoonosis, but it crosses the species barrier with difficulty and with little effect. Given the high occurrence of FMD in animals, its occurrence in man is rare (Prempeh *et al.,* 2001).

Due to the FMDV's sensitivity to pH the physiological buffer MES (with a pKa of 6.1) is well suited to destroy FMDV; it can buffer at a pH as low as 5 (Acharya *et al.,* 1990; Thompson & Bastos, 2004). The rate of virus inactivation is 90% per minute at pH 6.0 and 90% per second at pH 5. The virion disintegrates into its subunits (Bachrach, 1968).

A study described by Jooste *et al.,* (2003) showed that denuded embryos, produced *in vitro* (IVP), can be rendered free from FMDV by treating them with MES.

At present it is unknown what effects MES would have on semen as no research has been done to assess the viability of semen treated with MES.

2.2 The anatomy and physiology of the bovine reproductive tract

2.2.1 Reproductive organs of the bull

In broad terms, the anatomy of the buffalo correlates well with that of the domestic bovine (Hornsveld, 1996). Bertschinger (1996) found the anatomy of the reproductive organs of buffalo very similar to that of domestic cattle.

The male reproductive organs can be divided into three components namely the primary sex organs (testis), the accessory sex glands and ducts and the penis. The reproductive organs fulfill three functions which are the production of spermatozoa in the testis; the maturation, storage and transport of spermatozoa in the epididymal duct system and the deposition of semen in the female genital tract by means of the penis (Arthur *et al.* 1992).

The accessory sex glands of bulls are the ampullae, vesicular glands, prostate and bulbourethral glands. The end part of the vas deference dilates before entering the pelvic urethra and forms the ampullae, which acts as a storage space for sperm and contributes only minimally to the seminal plasma. The vesicular glands are paired glands divided into lobules, which secrete a considerable volume of a watery secretion. The prostate gland surrounds the urethra and consists of two parts: the body and the cryptic prostate. The bulbourethral glands are paired, small and round structures situated between the anus and urethra. These glands produce a large amount of watery secretion before coitus to flush the urethra (Ashdown & Hafez, 1993; Arthur *et al.,* 1992). Figure 2.2 shows a general view of the reproductive organs of a bull (Arthur *et al.,* 1992).

The secretions produced by the accessory sex glands are known as seminal plasma. The major function of seminal plasma is to increase the volume of the ejaculate. The seminal plasma contains the same electrolytes as blood plasma (Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Cl^{-}, HCO_3^{-}, HPO_4, $H_2PO_4^{-}$) and these are the chief contributors to osmolality. The carbohydrates (fructose, citrate, sorbitol and inositol) are the sources of energy in the semen. A variety of proteins occur in seminal plasma and function as important buffers and membrane protectants (Prins, 1998; Ross, 1998).

Figure 2.2: Diagram of the male genital organs of the bovine
a, testis; b, head of epididymis; c, body of epididymis; d, tail of epididymis; e, vas deference; f, vascular part of spermatic cord; g, ampulla of vas deferens; h, seminal vesicle; i, body of prostate; k, pelvic urethra surrounded by urethralis muscle; l, bulbourethral gland; m, bulbocavernosus muscle; n, crus penis; o, ischiocavernosus muscle; p, distal sigmoid flexure of penis, t, glans penis; s, retractor penis muscle; t, urinary bladder; u, pubic symphysis; v, rectum (From Blom & Christensen, 1947) (Arthur *et al.,* 1992).

2.2.2 Semen

The ultra-structure of the semen from buffalo was found to be very similar to that of domestic cattle (Ackerman *et al.,* 1994; Bertschinger & Cordell, unpublished data).

Semen is the male secretion from the reproductive organs containing the sperm cells. Sperm are the male gametes and are very specialized in terms of function and structure. The mature sperm cells are suspended in the seminal plasma during emission and ejaculation (Prins, 1998).

The spermatozoa are divided into three major components: head, midpiece and tail. The sperm head consists of the nucleus and the acrosome. The nucleus differs form nuclei usually found in somatic cells in that its volume is less and its chromatin more condensed. The nucleus contains the DNA which consists of only half of the normal DNA number and is combined with specialized proteins known as protamines. The anterior half of the nucleus is covered with an acrosome, which is a thin double-membraned cap-like structure. The acrosome contains a specific lipoprotein complex and hydrolytic enzymes. The hydrolitic enzymes break down muco-polysaccharides and assist the sperm to penetrate the zona pellucida surrounding the ovum during fertilization. Other enzymes contained in the acrosome may assist with the fusion of the sperm egg plasma membranes (Minette, 1999). The caudal part of the sperm head is indented, forming the implantation fossa. The implantation fossa is the attachment site of the tail (Barth & Oko, 1989; Setchell, 1991; Arthur, 1992; Hafez, 1993).

The movement of the sperm tail provides the locomotory force for the sperm. Movement of the sperm is required to allow the sperm to reach and penetrate the ovum at fertilisation. The sperm tail is morphologically subdivided into four parts: connecting piece, midpiece, principle piece and end piece. These four parts form a single functional structure. The tail consists of an inner axoneme which is enclosed in a helix of mitochondria in the region of the midpiece. The axoneme consists of nine peripheral microtubule doublets surrounding a central pair of singlets that occupy the middle part of the tail throughout the most of its length. The middle piece is covered with a mitochondrial sheath, which spirals around the axoneme (Millette, 1998). At the principle piece, a fibrous sheath replaces the mitochondrial spiral to cover the axoneme. Connecting fibers originating at the base of the sperm head cover the axoneme in the middle piece and part of the principle piece. The nine outer fibrils are probably the most important contractile parts generating contractions along the length of the tail resulting in sperm motility (Barth & Oka, 1989; Hafez, 1993;

Millette, 1998). The end piece of the tail is not encircled by a sheath and the nine outer fibrils are not present (Hafez, 1993; Millette, 1998). Figure 2.3 shows a diagram of the structure of a sperm cell (Arthur, 1992).

The sperm plasma membrane is a continuous membrane that covers the complete surface of the sperm from the most anterior part of the head to the end piece of the tail. The plasma membrane contains lipids and proteins which regulate certain aspects of sperm function and physiology (Millette, 1998).

During spermatogenesis meiotic cell division takes place resulting in a haploid chromosome number or half of that of somatic cells of the same species (Setchell, 1991; Arthur, 1992; Hafez, 1993).

2.2.3 Capacitation and acrosome reaction

Ejaculated mammalian sperm cells are not able to fertilise oocytes immediately and must first undergo a series of physiological changes called capacitation in the female reproductive tract before they acquire the ability to fertilise. (Chang 1951; Austin 1952; Yanagimachi 1994; Bedford, 1998). These changes include: an increase of membrane fluidity, an increase in intracellular calcium and a rise of intracellular pH, and migration of the acrosomal proteins, in order to accommodate the receptor sites on the zona pellucida. After capacitation spermatozoa show hyperactive movement, necessary for the sperm to reach the oocyte and to penetrate the zona pellucida during fertilisation. Capacitation is the final stage of sperm maturation and is crucial for the acrosome reaction and successive penetration of the zona pellucida and fertilisation of the oocyte. Complete capacitation of sperm is suppressed in the isthmus of the fallopian tube. Capacitation is only completed at the ampullary-isthmic junction close to the time of ovulation (Dunbar *et al.,* 1991; Crozet, 1993; Töpfer-Peterson *et al.,* 2000). Only capacitated sperm are able to recognise the binding sites on one of the three zona pellucida proteins.

The binding process commences with the acrosome reaction, which is a cascade of reactions (Töpfer-Peterson *et al.,* 2000; Ramalho-Santos *et al*., 2002; Visconti *et al.,* 2002; Cormier *et al.,* 2003). The acrosome reaction is described by Rathi as an irreversible, exocytotic event, characterised by a sudden increase in intracellular calcium concentration. The acrosome reaction is an indication that the capacitation process is finalised (Bazer *et al.,* 1993; Rathi, 2001).

During the acrosome reaction fusion of the outer acrosomal membrane and the overlying plasma membrane takes place (Rathi, 2001). The fusion and vesiculation of the acrosome results in release of hydrolytic enzymes (e.g. acrosin and hyaluronidase), which enables the sperm to create a tunnel along which the sperm burrows until it reaches the oocyte. Once the sperm reaches the oolemma it binds to it and the sperm is internalised (Bazer *et al.,* 1993; Rathi, 2001; Gordon, 2003).

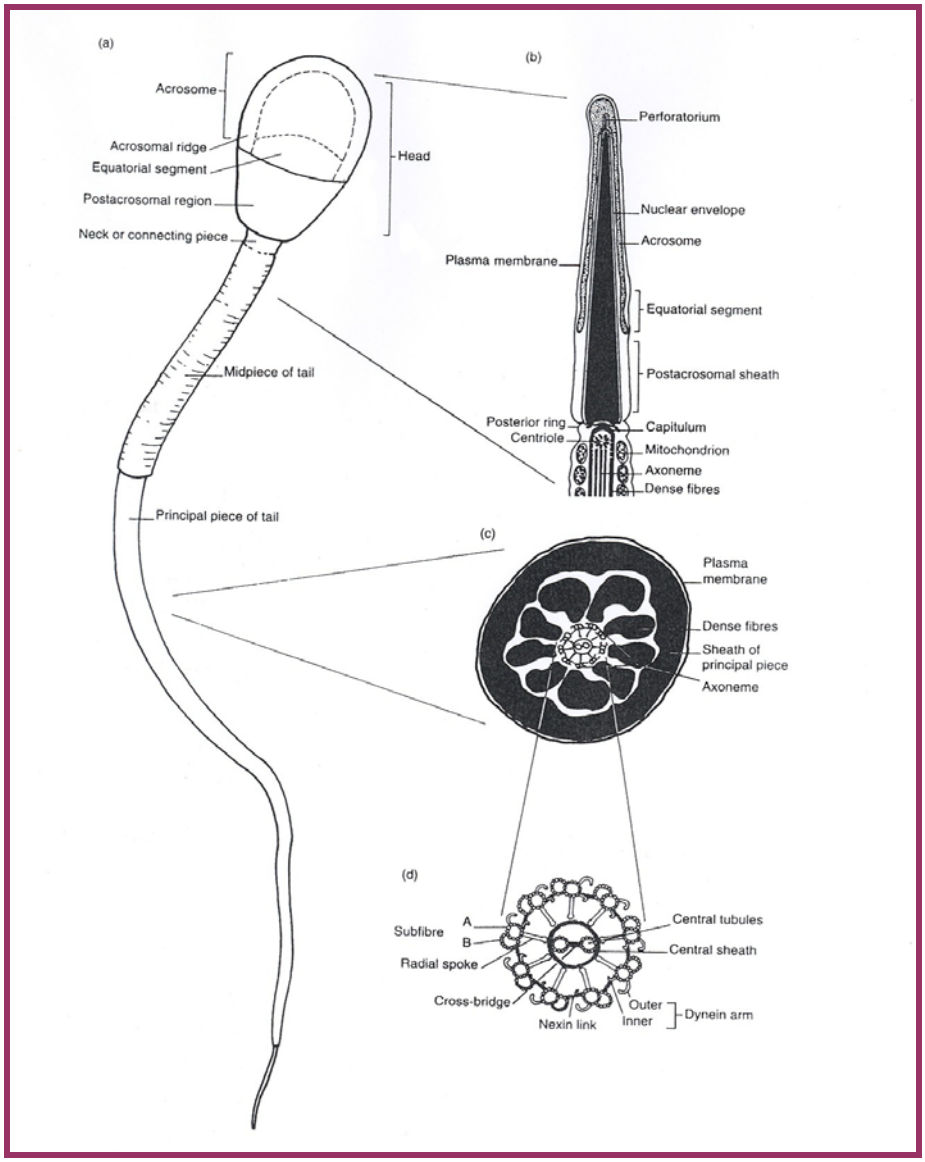

Figure 2.3: Diagram of the ultra-structure of a spermatozoon. a, Main structural regions as revealed by light microscopy. b, Generalized ultra-structural

features of the head and connecting piece. c, Ultra-structure of the proximal principle piece of the tail. d, Generalized detail of the ultra-structure of the axoneme of the tail. (Redrawn and adapted from Bedford and Hoskins (1990). (Arthur *et al.,* 1992).

2.3 Semen evaluation

For accurate semen evaluation, the sample must be examined as soon as possible after collection or where frozen semen is concerned after thawing (Barth & Oko, 1989). Warm, clean slides and fresh warm stain should be used during the evaluation (Hopper, 2006). The estimation of potential fertility of semen can be increased by examining a range of physical characteristics of the sample (Amman & Hammerstedt, 1993; Graham, 2001). In guidelines set by the Society for Theriogenology, minimum standards are set for these semen characteristics which should be met in order for a bull to pass a breeding soundness examination. The criteria for bull semen are as follows:

- a sperm concentration of over 500 million sperm per ml;
- linear sperm motility of more than 50%;
- normal sperm morphology of more than 80% (Society for Theriogenology, 2007; Ax *et al.,* 1993; Sprecher & Coe, 1996; Spitzer, 2000).

Recommended parameters to be evaluated during a semen analysis are the volume, sperm concentration, sperm motility, percentage live and dead sperm and sperm morphology (Ax *et al.,* 1993).

2.3.1 Thawing of frozen semen

A number of factors such as straw size, equilibration time, freezing rate and thaw rate can have an effect on the post-thaw quality of semen. Aamdal and Andersem (1968) found that an increase in thawing temperatures is positively related to the survival rate of sperm. Cochran *et al.,* (1984), found thawing of equine semen in 75° C water for 7 seconds to be superior to thawing in 37° C water for 30 seconds.

Wiggin and Almquist (1975a) concluded that higher thawing rates are superior to lower thawing rates as long as the thawing temperature does not exceed 76° C. In a number of studies by Arriola and Foote (1987) and Wiggin and Almquist (1975b) it was confirmed that higher thawing temperatures are superior to lower thawing temperatures for bull semen. The superiority of high thawing temperatures was confirmed by Nöthling and Shuttleworth (2005) who have found a higher percentage progressively motile sperm for frozen dog semen thawed at 70º C compared to frozen dog semen thawed at 37º C.

2.3.2 Staining technique

Eosin-nigrosin is a supra vital stain that has been used for decades to evaluate the viability of the plasma membrane and the acrosome integrity of sperm (Blom, 1950; Mayer *et al.,* 1951; Swanson & Bearden, 1951). The stain is used to indicate if the sperm membranes are damaged. The principle of this technique is stain exclusion, which means that the eosin cannot penetrate through an intact membrane, but any sperm will take up the stain if the membranes are damaged. Nigrosin is used as a background stain to assist the reading of the stained and non-stained sperm. The percentage of sperm with intact plasma membranes should correlate with the percentage of progressively motile (prm) sperm (Björndal *et al.,* 2003; Brito, 2003).

2.3.3 Sperm motility

Mature sperm are stored in an inactive state within the *cauda epididymis*, but show an immediate burst of motility when released from this environment at the time of ejaculation. A range of mechanisms have been described to explain the commencement of motility at ejaculation. Factors such as ion movement, changes in surrounding medium viscosity, alkalinisation of the cytoplasm, the presence of HCO_3^- and increases in intracellular cyclic adenosine monophosphate (cAMP) are described (Jones & Murdoch, 1996).

One of the unique features of spermatozoa is their ability to move. The assessment of sperm motility is a subjective visual assessment of motile sperm in a semen sample. The viability of the sperm and the quality of movement is estimated. The percentage of progressive motile (prm; sperm swimming rapidly forward in a straight line), aberrantly motile (abm) and immotile (imm) sperm respectively are evaluated (Nöthling & Shuttleworth, 2005). The minimum recommended linear progressive motility for frozen-thawed bull semen as set by the Society for Theriogenology is 30% (Spitzer, 2000; Hopper, 2006; Society for Theriogenology, 2007). Daza (1994) also found that a minimum limit of at least 30% prm sperm, for frozen-thawed bull semen, is considered acceptable for use in AI programmes.

Due to the high sperm concentration of ejaculated bull semen it is necessary to extend semen with a semen extender prior to the evaluation of sperm motility (Ax *et. al.* 1993). Before the freezing process semen samples are diluted to ensure a concentration of at least 15 x 10^6 sperm cells per mL (Hafez, 1993). Sperm cells are very vulnerable to environmental conditions such as extreme heat or cold and care must be taken to maintain the semen sample at an acceptable temperature prior to examination. Due to the subjective character of this examination, some variability in analysis of samples is found. The use of computer assisted sperm analysis (CASA) systems has made it possible to reduce the variability to some extent. These systems make use of image processing software that detects and tabulates the tracks of motile and immotile sperm. Although very expensive, these systems are used regularly in the semen evaluation process in most laboratories (Budworth *et al.,* 1988; Jasco, 1992).

If the evaluation of sperm motility is done correctly the percentage of unstained sperm on a semen smear stained with a vital stain, will correlate with the visual estimates of prm sperm (Brito *et al.,* 2003).

Cryopreservation causes irreversible damage to the sperm membranes that results in either cell death or capacitation-like changes in the plasma lemma (Hammerstedt

et al., 1990).The sperm cells cannot repair themselves from damage which renders them infertile (Graham, 2001). The evaluation of sperm motility is an important parameter to determine the extent of damage to sperm due to the cryopreservation method. In several studies linear motility of sperm could be significantly correlated with field fertility (Kjaestad e*t al.,* 1993; Farrell *et al.,* 1998; Januskauskas *et al.,* 2001; Rodriques-Martinez, 2000).

Valcárcel *et al.,* (1997) found that at least 30% of plasma membranes from frozen-thawed motile ram spermatozoa were damaged. According to the authors the damaged spermatozoa rapidly lose their motility at 37° C and would thus be non-functional in vivo.

The high unsaturated fatty-acid content of sperm makes them susceptible to peroxidation in the presence of oxygen. Therefore an extended time period of exposure to air is detrimental to sperm motility (White, 1993). Peroxidation is a destructive process, which is most active in damaged or less motile sperm and the toxic products formed during peroxidation are harmful to other spermatozoa (Shannon & Curson, 1972; Jones & Mann, 1973; Jones & Mann, 1977; Aitken & Clarkson, 1988; Sexton, 1988; Slaweta *et al.,* 1988).

To prepare a sample for evaluation of motility, a small drop (4 µl) of diluted semen is placed on a pre-warmed (37º C) cover slip. The cover slip containing the drop of semen is inverted onto a pre-warmed (37º C) microscope slide. The semen will be flattened under the cover slip onto the microscope slide. Ten different fields are evaluated, starting from side of the cover slip to the centre. The mean of the 10 values are taken as the prm, abm and imm sperm respectively (Nöthling & Shuttleworth, 2005).

2.3.4 Plasma membrane integrity (sperm viability)

The plasma membrane is one of the areas where lesions occur most often during the freezing and thawing of semen, resulting in plasma membrane disorders and cell death (Hammerstedt *et al.,* 1990; Martin *et al.,* 2004). The percentage of sperm with intact and damaged plasma membranes can be assessed on dried semen smears stained with a vital stain such as eosin-nigrosin. The plasma membrane of live sperm are intact and therefore keep the large stain molecules of the eosin stain out of the cell, whereas the plasma membrane of sperm with damaged plasma membranes will take up the stain. Nigrosin is used as a back-ground stain to facilitate the visualisation of sperm (Dott & Foster, 1972; Rodriguez-Martinez, 2000; Nöthling, 2000; Graham, 2001; Brito *et al.,* 2003).

2.3.5 Acrosomal integrity

Even if a sperm is viable and has the ability to move in a linear progressive way, it will not be able to fertilize an oocyte if the acrosome is damaged or lost. The acrosomal membranes are the most labile membranes of the sperm as it is pre-programmed to undergo membrane fusion during capacitation. According to Graham (2001) the acrosomal membranes are the most likely membrane to be damaged during handling, freezing and thawing.

On stained semen smears acrosomes are classified as intact, damaged or lost. If the acrosome remains in contact with the sperm head it is classified as intact. If the acrosomal membrane was damaged or separated from the sperm head it is classified as damaged. Sperm with no acrosome is classified as a sperm with a missing acrosome (Spindler *et al.,* 2004).

2.3.6 Longevity of sperm

The survival of sperm in the isthmus is linked to immobilisation. It is known that ejaculated spermatozoa can survive for many days in the bitch and in women and for weeks in the female hare. The survival of ejaculated sperm relies on specific environmental conditions such as ion movements; changes in the viscosity of the surrounding medium; cytoplasmic alkalinisation; increases in intracellular cAMP (cyclic adenosine monophosphate); the presence of high potassium content (found in the male genital tract); the presence of HCO_3^-; high glycine content which maintains respiratory activity; and the deposition of certain proteins secreted by the endometrial and tubal mucosa (Fournier-Delpech & Thibault, 1993; Jones & Murdoch, 1996).

Shortened longevity of sperm may play an important role in failed fertilisation. The effects of cryopreservation on physical characteristics of frozen thawed semen were mostly evaluated immediately after thawing of the semen. More recent studies showed that the evaluation of fresh and frozen-thawed semen is more significant if the semen is stored at a certain temperature for a number of hours and sperm parameters are repeatedly evaluated over time.

Margolin (1943) found a significant relationship between longevity of sperm (duration of motility), fertility and the conception rate of bull semen.

In a study done by Ponglowhapan (2004) freshly ejaculated dog semen was mixed with egg-yolk-Tris extender containing glucose, fructose or both. Glucose and fructose had an influence on motility, but fructose maintained higher sperm motility than the glucose or the glucose-fructose mixture.

Previous studies showed that sperm is susceptible to shifts in temperature (Drobnis *et al.,* 2000). Johnson and Prien (2003) showed a significant increase in the viability, motility and acrosome intact cells of dog semen samples collected in warmed semen extender Androhep^{TM} compared to a sample that was collected with the traditional method (left dry and no special procedures were taken).

Correa and Zavos (1995) evaluated slow and rapid dilution methods for frozen-thawed bull semen. With the rapid dilution method, the dilution medium was added in a single step, whereas with the slow dilution method the dilution medium was added at a rate of 0,1 mL per minute via a drop-wise method. An equal motility rate was found directly after thawing, but a significant difference was found after 2 hours of incubation at 37º C. These findings indicate that different dilution methods for frozen-thawed bull semen that result in similar post-thaw motility, can show differences when re-evaluated some time after thawing.

Muiño *et al.,* (2007) compared two egg yolk-free semen extenders to a Tris-egg yolk diluent and found that the Tris-egg yolk diluent showed higher sperm survival immediately and nine hours after thawing.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bull semen used

Frozen bovine semen was obtained from Taurus Co-operative (Irene, South Africa, 0062). Taurus Co-operative is a local primary agricultural co-operative which produces and supplies frozen bull semen for the South African market. Semen was frozen in 0.25 mL French straws at a concentration of 80 x 10^6 sperm cells per mL (Havinga, 2006). Frozen semen from four different breeds was used for this trial: five Frieslands, three Jerseys, one Ayrshire and one Simmentaler. Semen straws from two different batches were used from each bull. All data was recorded on a data sheet (Appendix 1).

3.2 Preparation of solutions

3.2.1 Preparation of the MES solution used for the trial

A 1 M MES solution was prepared by dissolving 0.848 mg MES in 10 mL distilled water. An aliquot of 2.5 mL of this MES solution was added to 97.5 mL Sabax® physiological saline. The pH of the solution was adjusted to 5 by adding 1 N hydrochloric acid (HCl).

3.2.2 Preparation of the phosphate buffered saline (PBS) used for the trial

The PBS solution used for the trial was prepared by adding different chemicals to 500 mL Sabax® physiological saline (see Table 3.1).

Table 3.1: Chemicals used for the preparation of the phosphate buffered saline used for the trial

Chemical	Sigma-Aldrich catalogue number	Gram per 500 mL Sabax® physiological saline
NaCl	S-5886	3.33
KCl	P-5405	0.119
$NaHCO_3$	S-4019	1.045
$NaH_2PO_4.1H_2O$	S-5011	0.0275
$MgCl_2.6H_2O$	M-2393	0.05
$CaCl.2H_2O$	C-7902	0.147
HEPES	H-0763	1.2
Phenol Red	P-5530	0.05
Sodium Pyruvate	P-3662	0.0275
Sodium Lactate	L-4263	0.8844
Gentamycin	G-1264	0.0125

The pH of the solution was adjusted to 7.4 by adding 1N hydrochloric acid or 1 N sodium hydroxide. The solution used for the trial had an osmolality of 310.

3.3 Experimental procedures

The experimental procedures for the control (Co) and MES-treated (Tr) samples are shown in Fig 3.1.

Figure 3.1 Flow diagram indicating the experimental procedures for the control and treated samples

3.3.1 Thawing of semen

Three semen straws from each batch were thawed by plunging the straws into a water bath at a temperature of 38º C for at least 25 s (Gaillard & Kupferschmied, 1982). The temperature of the water in the water bath was controlled by a thermostat, but the temperature was checked before use. The contents of the three straws were emptied into a pre-heated 12 mm (millimeter) x 75 mm plastic test tube. The sample was split into two aliquots. The one aliquot was used as control (Co) whilst the other aliquot was exposed to MES treatment (Tr). After thawing the semen was maintained at a constant temperature of 37º C.

3.3.2 Control samples (Co)

The control samples were incubated and evaluated at the same time as the treated samples. The pH and osmolality of the control samples are shown in Table 3.2.

Table 3.2: pH and osmolality of solutions used in the trial

	MES	PBS	Frozen-thawed semen	0.25 mL Frozen-thawed semen + 2.25 mL MES	0.25 mL Frozen-thawed semen + 2.25 mL MES + 10 mL PBS
pH	5.00	7.40	7.50	5.72	6.86
mOsm	327	310	305	301	339

3.3.3 Exposure of treatment samples to MES (Tr)

For all treatment samples, 2.25 mL of the MES solution was added to 0.25 mL semen. After an exposure of 60 sec to the MES solution, 10 mL of the PBS solution was added to the sample. The pH and osmolality of the treatment samples are

shown in Table 3.2. The treatment samples were centrifuged at 300 G for 10 min. The supernatant was removed using a Venturi pump connected to a Pasteur pipette and the pellet re-suspended with 0.5 mL IVF TALP based medium (Arlotto *et al.,* 2001). Chemicals used for the preparation of the IVF TALP based medium are listed in Table 3.3.

Table 3.3: Chemicals used for the preparation of the IVF TALP based medium (pH 7.4, osmolality 275-285)

Chemical	Sigma-Aldrich catalogue number	Gram/ml per 500 mL TALP based medium
NaCl	S-5886	3.325 g
KCl	P-5405	0.118 g
NaH_2PO_4	S-5011	0.021 g
$CaCl_2.2H_20$	C-7902	0.147 g
$MgCl_2$	M-2393	0.051 g
Lactic Acid (Na-Salt)	L-4263	0.95 mL
$NaHCO_3$	S-5761	1.050 g
Phenol red	P-5530	0.005 g
Sabax® H_2O		490 mL

3.3.4 Evaluations of sperm parameters performed on control (Co) and treated (Tr) semen samples

3.3.4.1 Sperm motility

A Triladyl semen extender was prepared as described by Nöthling and Volkmann (1993). The Triladyl semen extender consisted of 20 mL Triladyl™ concentrate

(Minitüb, GmBH, Tiefenbach, Germany), 60 mL deionised water, 20 mL egg yolk and 0.5 ml Equex STM paste (Nova Chemical Sales, Scituate, MA, USA). Triladyl™ concentrate is a patented freezing diluent containing Tris, citric acid, glycerol, fructose, tylosin, Spectinomycin, Gentamycin and Lincomycin. Equex STM paste contains 2.2 % m/m triethanolamine lauryl sulphate.

Four drops (16 µL) of Triladyl semen extender were placed on a clean, dust free cover slip (22 mm x 22 mm). A small drop (4 µL) of semen was added to this. The semen was mixed with the Triladyl semen extender. A small drop of the diluted semen (about 5 µL) was placed on a 22 mm x 22 mm cover slip. The cover slip was inverted and slowly lowered over a microscope slide until the hanging droplet touched the microscope slide. The cover slip was allowed to fall onto the slide, flattening the hanging droplet into an evenly spread thin film without air bubbles. The small drop size ensured that no excess of the fluid flowed from underneath the sides of the cover slip (Hopkins & Evans, 2003).

The slide was immediately placed on a warm stage at 37º C. A phase-contrast microscope at 200 x magnification was used for the evaluation. The percentage progressively motile and the percentage aberrantly moving sperm were estimated subjectively by eyeball assessment. The percentage imm sperm was calculated by the subtraction of the percentage prm and the percentage abm sperm from 100%.

A series of 10 adjacent microscope fields were assessed, the first field being approximately at the centre of the cover slip and the tenth at the edge of the cover slip. The mean of the 10 values were taken as the percentage prm, abm and imm sperm respectively (Nöthling & Shuttleworth, 2005).

3.3.4.2 Plasma membrane integrity (sperm viability)

The percentages of spermatozoa with intact and damaged plasma membranes were determined by means of a thin smear that was examined microscopically. The eosin-nigrosin stain was prepared by adding 2 g eosin and 5 g nigrosin to 100 mL of a water based buffer with a pH of less that 6.8 and an osmolality of 293. After adding eosin and nigrosin the solution was sonicated and filtered. The eosin-nigrosin stain had a pH of 8.49 and an osmolality of 420 (Bertschinger, 2008).

Using a Pasteur pipette, four drops of warm eosin-nigrosin stain were placed in the centre of a pre-warmed microscope slide. One drop of semen was mixed with the stain for about 1 min. A drop of the semen mixture was placed on another pre-warmed microscope slide. The back end of a spreader slide was lowered over the edge of the drop until the drop spread along the edge of the spreader slide. The spreader slide was swept at a moderate rate across the entire microscope slide. This method ensured that the drop was pulled and not pushed; minimizing possible damage to the sperm cells (Hopper, 2006). The smears were immediately dried with a hair dryer and mounted with Entellan® (Merck KgaA, Darmstadt, Germany, cat no: 1.07961.0500) rapid mounting medium, which is a xylene containing resin.

A phase-contrast microscope at 1000 x magnification was used for the evaluation. A total of 200 sperm were examined to determine the percentage of sperm with intact and damaged plasma membranes. Spermatozoa were classified as follows:

- spermatozoa that stained partially or complete purple were considered to be sperm with damaged plasma membranes ;
- unstained spermatozoa were considered to be sperm with intact plasma membranes (Brito, 2003)

Sperm viability of both the Co and Tr samples were evaluated and the results obtained were compared both between the samples and between the breeds. Figure 3.2 indicates the difference in appearance of the live and dead sperm.

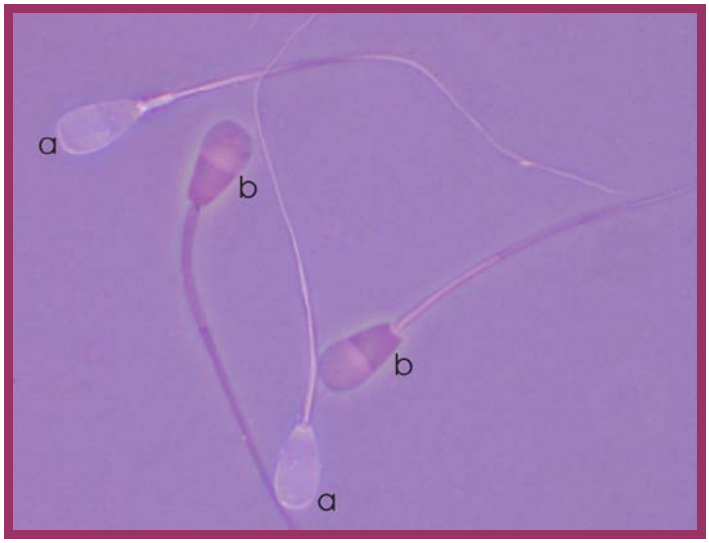

Figure 3.2: Semen smear stained with eosin-nigrosin for evaluation of the plasma membrane demonstrating sperm with: a, intact plasma membrane and b, damaged plasma membrane

3.3.4.3 Acrosomal integrity

The same thin semen smears stained with eosin-nigrosin stain that were used to evaluate the percentage sperm with intact and damaged plasma membranes, were also used to evaluate the acrosomal integrity of the sperm. Acrosomes were classified as follows:

- acrosomes that had a smooth appearance and remained intact with the sperm head were classified as normal;
- acrosomes with a ruffled appearance and those separated from the head were classified as damaged or degenerated; and

- sperm with a shoulder visible at the equatorial region were classified as sperm with lost acrosomes (Spindler *et al.,* 2004).

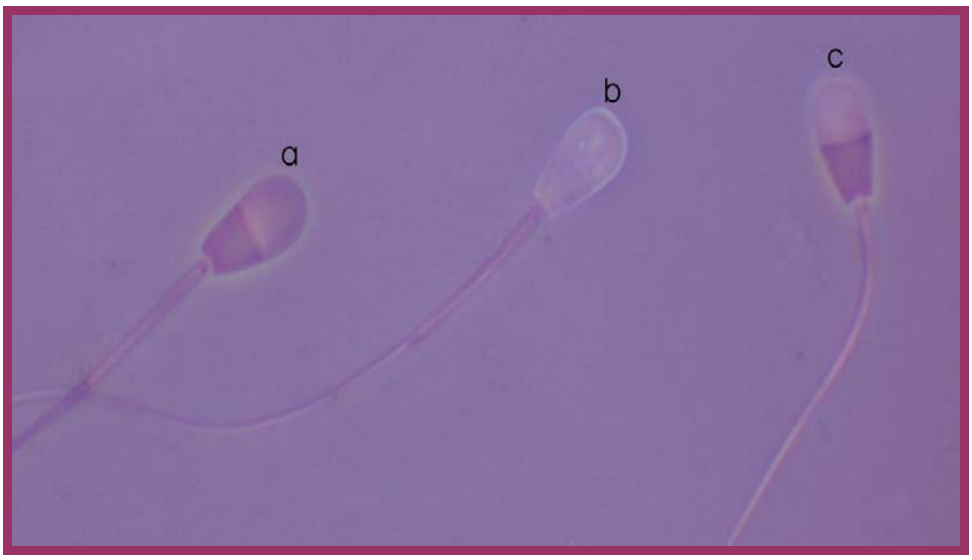

Figure 3.3: Semen smear stained with eosin-nigrosin for evaluation of the acrosomal membrane demonstrating sperm with: a, reacting acrosome; b, intact acrosome and c, lost acrosome

3.3.4.4 Longevity of sperm

The Co and Tr semen samples were kept in a water bath at 37º C and the percentage prm, abm and imm sperm determined immediately after thawing and evaluation (t_0), as well as one (t_1) and two hours (t_2) later. The change in prm, abm and imm sperm over time for the Co and Tr samples were compared.

3.4 Statistical analysis

Semen from two different batches from the ten bulls used in this trial was evaluated. Variables from each batch were evaluated for the Co and Tr (MES exposed) semen. For each measured variable the average value of the two batches was used for the statistical analysis.

Statistical analyses for plasma membrane and acrosomal membrane integrity were performed with the computer program NCSS 2001 (NCSS, 320 North 1000 East, Kazsville, Utah 84037, USA). The coefficient of correlation for the variables was compared using a paired t-test and significance was set at $p < 0.05$.

In addition a “Repeated Measure One Way” ANOVA was used to determine motility over time and longevity of sperm (a change in motility over time) of the Co and Tr samples. The Geisser-Greenhouse ε-correlations were applied to determine the level of significance. Significance was set at $p < 0.05$.

CHAPTER 4

RESULTS

4.1 Introduction

The effect of MES treatment on the motility, plasma membrane integrity, acrosomal membrane integrity and longevity of sperm are discussed below. Detailed experimental results are shown as Tables in Chapter 4 and are graphically presented as Figures in Appendix 2. Significant findings are graphically presented as Figures 4.1- 4.6 in Chapter 4.

4.2 Motility

Immediately after thawing (t_0) and one hour later (t_1) no significant differences were seen between Co and Tr samples for prm, abm and imm sperm; two hours after thawing (t_2) a significant difference ($p = 0.000001$) was seen between the Co and Tr samples for abm sperm (Fig 4.1 and Table 4.1).

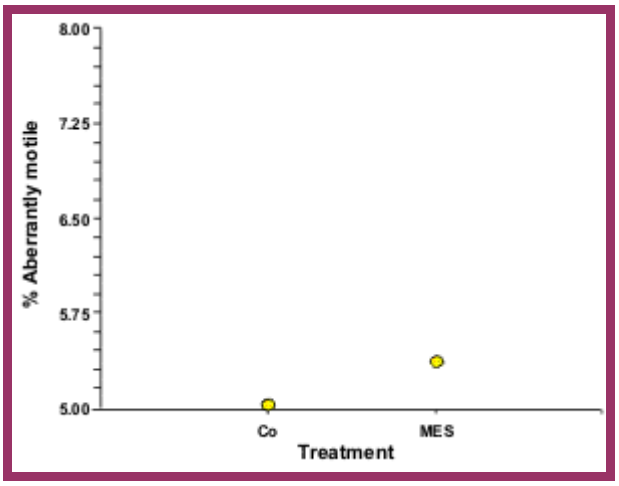

Figure 4.1: Influence of treatment on % aberrantly motile sperm (% abm) 2 hours (t_2) after thawing

At times t_0 and t_1 no effect of breed was found for prm, abm and imm sperm between the Co and Tr samples; at t_2 a significant breed effect was found for abm sperm ($p = 0.000001$; Fig 4.2).

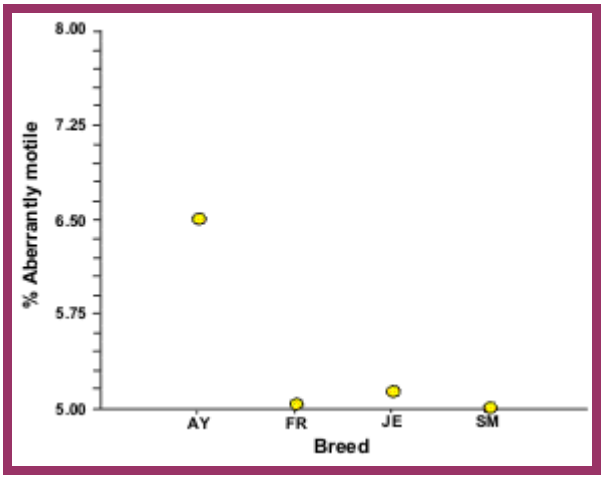

Figure 4.2: Influence of breed (AY, Ayrshire; Fr, Friesland; JE, Jersey; SM, Simmentaler) on the % aberrantly motile (abm) sperm 2 hours (t_2) after thawing

For the combined effect of treatment and breed no significant differences were seen between Co and Tr samples at t_0 and t_1. A significant difference however was seen for the abm sperm at t_2 ($p = 0.000001$; Fig 4.3).

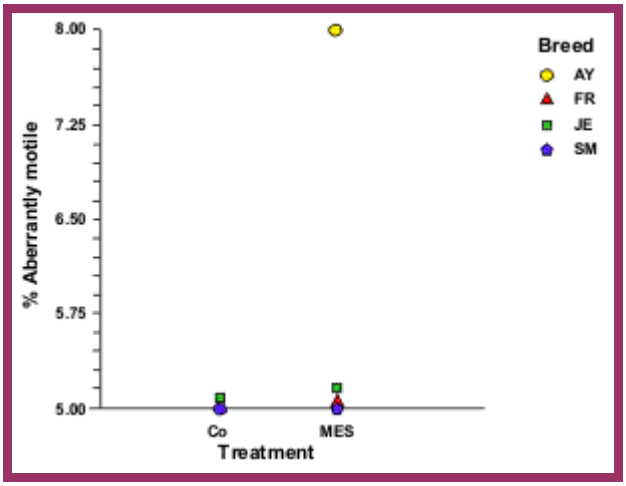

Figure 4.3: Influence of treatment and breed (AY, Ayrshire; Fr, Friesland; JE, Jersey; SM, Simmentaler) on % aberrantly motile (% abm) sperm 2 hours (t_2) after thawing

The influence of MES treatment on the motility of sperm is graphically presented in Fig 7.1 to Fig 7.27 of Appendix 2 and is summarised in Table 4.1.

Table 4.1: Influence of MES-treatment (Tr) on motility, plasma membrane viability and acrosomal integrity of sperm; the latter two are indicated as total numbers out of 200 spermatozoa

Parameter		Control (Co) Mean (± SD)	Treated (Tr) Mean (± SD)	P-value
Motility				
Progressively motile	t_0	55.13%[a] (± 6.05%)	55.95%[a] (± 6.05%)	p = 0.135
	t_1	52.10%[a] (± 5.55%)	50.99%[a] (± 5.55%)	p = 0.586
	t_2	45.48%[a] (± 9.51%)	40.90%[a] (± 9.51%)	p = 0.270
Aberrantly motile	t_0	5.35%[a] (± 0.64%)	5.20%[a] (± 0.64)	p = 0.556
	t_1	5.00%[a] (± 0.57%)	5.25%[a] (± 0.57%)	p = 0.509
	t_2	5.03%[a] (± 14.67%)	5.38%[b] (± 14.67%)	p = 0.000001
Immotile	t_0	39.05%[a] (± 5.17%)	40.25%[a] (± 5.17%)	p = 0.144
	t_1	43.20%[a] (± 5.72%)	44.90%[a] (± 5.72%)	p = 0.556
	t_2	50.43%[a] (± 8.49%)	52.90%[a] (± 8.49%)	p = 0.445
Plasma membrane				
Intact		130.15[a] (± 19.58)	104.30[a] (± 19.58)	p = 0 .088
Damaged		70.05[a] (± 18.85)	96.10[a] (± 18.85)	p = 0.077
Acrosome				
Intact		134.90[a] (± 20.19)	100.20[b] (± 20.19)	p = 0.012
Reacting		43.80[a] (± 17.03)	81.55[b] (± 17.03)	p = 0.004
Lost		22.30[a] (± 10.77)	18.65[a] (± 10.77)	p = 0.514

Similar superscripts between the results for Co and Tr samples within the same row indicate no significant difference; different superscripts within the same row indicate a significant difference ($p < 0.05$).

4.3 Plasma membrane integrity (sperm viability) at t_0

No significant difference was found for the number of unstained (live) sperm and stained (dead/damaged) sperm between the Co and Tr samples. For the effect of breed and the combined effect of treatment and breed no significant differences were seen between the Co and Tr samples for the number of sperm with intact plasma membranes and sperm with damaged plasma membranes. The influence of MES-treatment on plasma membrane viability is graphically presented in Figures 7.28 to 7.33 in Appendix 2 and is also summarised in Table 4.1.

4.4 Acrosomal integrity at t_0

A significant difference was found for the number of intact and reacting acrosomes between the Co and Tr samples (Figures 4.4 and 4.5), whereas no significant difference was found for the number of lost acrosomes between the Co and Tr samples.

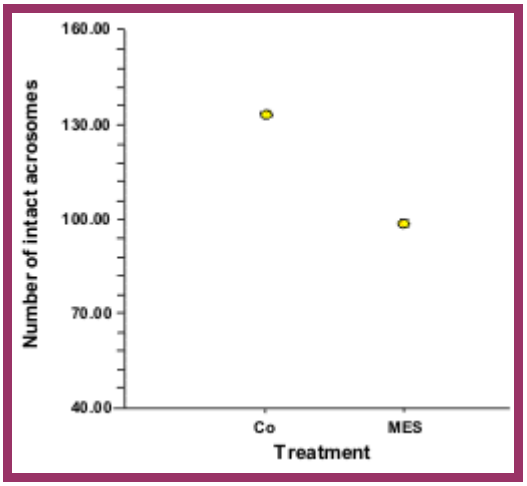

Figure 4.4: Influence of treatment on the number of sperm (n=200) with intact acrosomes

No significant breed effect or combined effect of treatment and between Co and Tr samples were found for the number of intact, reacting or lost acrosomes. Figures 7.34 to 7.42 in Appendix 2 graphically present the influence of MES treatment on acrosome integrity and the findings are also summarised in Table 4.1.

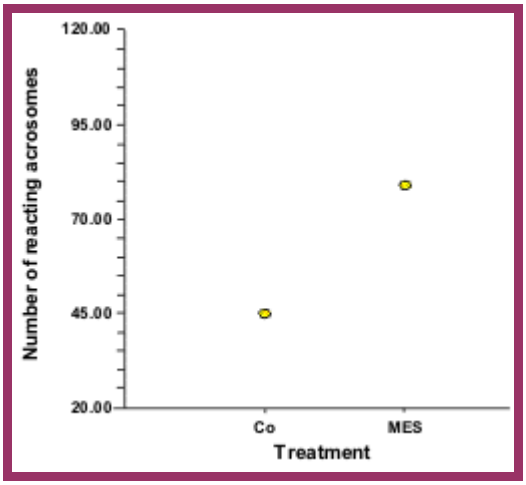

Figure 4.5: Influence of treatment on the number of sperm (n=200) with reacting acrosomes

4.5 Longevity of sperm at t_0, t_1 and t_2

4.5.1 Progressively motile sperm

No significant difference for sperm longevity was found initially between the Co and Tr samples (Fig 7.44 in Appendix 2). However, a significant difference was found in progressive motility over time ($p = 0.000001$) for both the Co and Tr samples as shown in Fig 4.6. No significant combined effect of treatment and time on longevity of prm sperm was found as graphically presented in Figures 7.48 and 7.51 in Appendix 2.

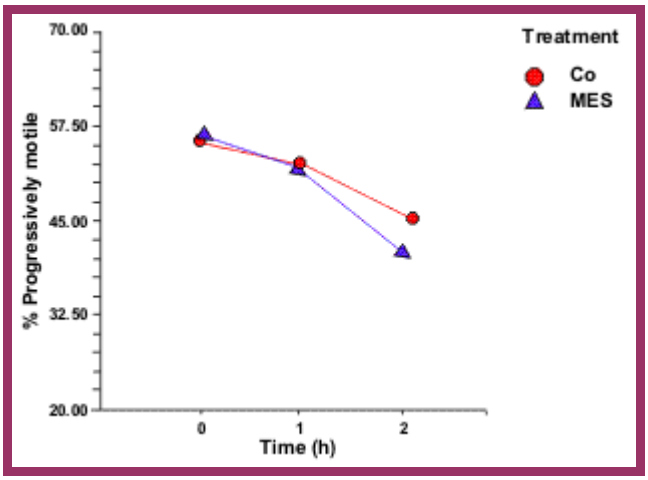

Figure 4.6: Influence of treatment and time on the longevity of sperm measured by the change in % progressively motile spermatozoa

The influence of MES-treatment and time on the longevity of prm sperm are summarised in Table 4.2 and graphically presented in Appendix 2 (Figs 7.44, 7.45, 7.48 and 7.51).

Table 4.2: Influence of MES-treatment (Tr) on longevity of progressively motile (prm), aberrantly motile (abm) and immotile (imm) sperm for the Co and Tr samples

	Progressively motile			Aberrantly motile			Immotile		
Time	t_0 Mean (± SD)	t_1 Mean (± SD)	t_2 Mean (± SD)	t_0 Mean (± SD)	t_1 Mean (± SD)	t_2 Mean (± SD)	t_0 Mean (± SD)	t_1 Mean (± SD)	t_2 Mean (± SD)
Co	55.13 (5.22)	52.10 (5.22)	45.48 (5.22)	5.35 (0.50)	5.00 (0.50)	5.03 (0.50)	39.05 (6.22)	43.20 (6.22)	50.43 (6.22)
Tr	55.95 (5.22)	51.00 (5.22)	40.90 (5.22)	5.20 (0.50)	5.25 (0.50)	5.38 (0.50)	40.25 (6.22)	44.90 (6.22)	52.90 (6.22)

4.5.2 Aberrantly motile sperm

No significant difference in longevity was found for the abm sperm between the Co and Tr samples, over time or for the combined factor of treatment and time. The influences of MES-treatment, time and the combined factor of treatment and time on the longevity of abm sperm are graphically presented in Figures 7.51, 7.54 and 7.55 in Appendix 2 and are also summarised in Table 4.2.

4.5.3 Immotile sperm

No significant difference was found for the number of imm sperm between the Co and Tr samples or for the combined factor of treatment and time, whereas a significant difference ($p < 0.001$) was found for the number of imm sperm over time as shown in Fig 4.7

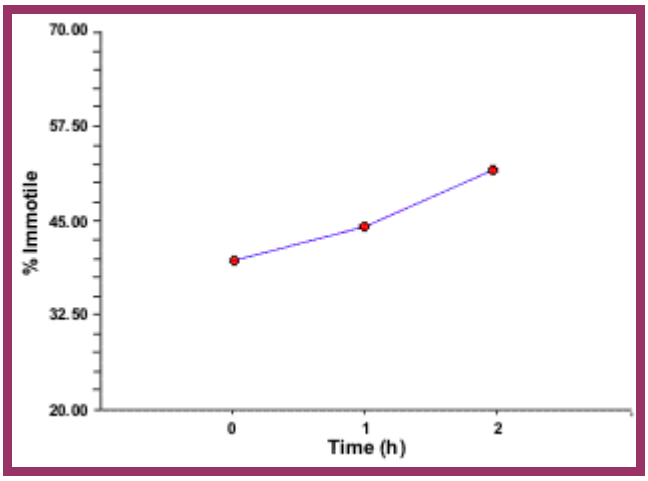

Figure 4.7: Influence of time on the % immotile sperm

The influences of MES-treatment, time and the combined factor of treatment and time on the number of immotile sperm are graphically presented in Figures 7.64, 7.65 and 7.71 in Appendix 2 and the results are also summarised in Table 4.2.

CHAPTER 5

DISCUSSION

5.1 Introduction

FMD is an important disease affecting buffalo in Southern Africa. The KNP in RSA is regarded as a FMD infected area. FMD control zones exist around the endemic areas to control the spread of FMD. Movement of buffalo from the endemic area is governed by strict legislation making it very difficult to reproduce the valuable genetic material of the buffalo in the KNP (Animal Diseases Act, 1984; Bastos, 1998; Leforban & Gerbier, 2002; National Department of Agriculture, 2008). Assisted reproductive techniques can be a useful tool in obtaining the sought after genetic material from FMD infected buffalo from the KNP to produce disease-free buffalo.

FMDV is very sensitive to pH and can be inactivated by a pH <6.0 and >9.0. MES with a pH of 5 can destroy FMDV highly efficiently (Acharya *et al.,* 1990; Thompson & Bastos, 2004). In a study by Jooste (2004) it was shown that IVP embryos exposed to FMDV could be cleared of infective FMDV with MES (Jooste *et al.* 2003; Jooste, 2004).

Frozen-thawed bull semen was used as a model for African buffalo semen as the morphology and physiology are similar (Bertschinger, 1996). The aim of this study was to determine if frozen-thawed bull semen could be treated with the acidic physiological buffer MES, without any detrimental effect to the motility, plasma membrane integrity, acrosomal membrane integrity and longevity of sperm.
In this study frozen-thawed semen was used because:

- Semen from the endemic areas is supposed to be used within the area. If it can be proven that semen treated with MES renders it consistently free of FMDV and of an acceptable quality for AI, the semen could be used to inseminate animals outside the controlled areas.

- There may be frozen semen from domestic bulls with superior genetic traits, with unknown FMD status, in FMD endemic areas. Semen can be collected and frozen at the site of collection. If found to be FMD positive, the semen can then be treated with MES at a later stage and used for insemination.

- Buffalo are often culled as part of population management programmes. During a routine culling operation, sperm from healthy, mature buffalo bulls can be collected from the cauda epididymis immediately post–mortem. Sperm can be collected by flushing the epididymis with air in a retrograde direction. The sperm can then be diluted with a semen extender and frozen (Herold 2003). Where the FMD status of buffalo is unknown, semen can be frozen immediately after collection at the culling site. The semen can then be thawed at a later stage and if found to be FMD positive, treated with MES.

5.2 Motility

The frozen-thawed semen from the different bulls used in this study differed in the percentage of their cells that survived freezing. The motility of all the frozen thawed semen immediately after thawing, however, complied with the recommended minimum of 30% progressive motility, as set out by the Society for Theriogenology (Society for Theriogenology, 2007).

Defects of the sperm tail are often associated with poor sperm motility. Sperm with tail defects commonly show abnormal movement and it is unlikely that such sperm will be able to reach the ovum or to penetrate the zona pellucida of the ovum during

fertilisation. This would result in failure to initiate a zona reaction, necessary for fertilisation (Barth & Oko, 1989).

In this study the majority of abm sperm observed showed tight circular movement, which is often associated with cold shock. The susceptibility to cold shock in sperm is reportedly associated with one or more of the following:

- the migration of the cytoplasmic droplet which occurs during the maturation of sperm;
- the modification in plasma membrane constituents, such as phospholipids; and
- the surface properties linked to the acquisition of motility and the fertilising ability in the epididymis (White, 1993).

The thawed semen samples, microscope slides, cover slips, eosin-nigrosin stain, semen extender and pipettes used for the handling and examination of all samples were however maintained at a constant temperature of 37 °C. A significant difference between the Co and Tr samples for the abm sperm was furthermore only found at t_2. As a result, cold shock can be excluded as a cause for the observed significant difference. The fact that no significant difference was found for the plasma membrane integrity between the Co and Tr samples as discussed under 5.2 further excludes cold shock as the cause of the increase in abm sperm at t_2.

The frozen-thawed semen samples were exposed to air for the duration of the evaluations. Thus, it is likely that the peroxidation process which is most active in damaged or less motile sperm was responsible for the increase of abm sperm at t_2. The fact that toxic products are formed during peroxidation that is harmful to sperm is also a contributing factor for the increase of abm sperm at t_2.

Semen quality traits of beef bulls are generally worse than those of dairy bulls and differences between cattle breeds have also been reported (Pileckas *et al.,* 2007*;* Den Daas *et al.,* 1998; Correa *et al.,* 1997). Individual variation between bulls was found in this study. Although semen from two batches of four different breeds was used, the difference in the results between breeds was not regarded as meaningfull, due to the small number of bulls of the different breeds used for this study (n =1 for Ayrshire, n = 1 for Simmentaler, n = 3 for Jersey and n = 5 for Friesland). Similarly, although a statistical significant difference for the combined effect of treatment and breed was calculated for abm sperm at t_2 the results can also not be interpreted as significant because the breed is only represented by one bull and the apparent difference in breed might thus be ascribed to an individual factor.

5.3 Plasma membrane integrity (sperm viability) immediately after thawing

As no significant difference was found between the number of sperm with intact and damaged plasma membranes immediately after thawing between the Co and Tr samples it can be assumed that MES treatment did not have a significant detrimental effect on the plasma membrane of the sperm. The sperm plasma membrane covers the entire sperm from the anterior part of the head to the most posterior part of the tail. If MES had a damaging effect on the plasma membrane we would have expected to find a significant difference in the motility for both the prm and abm sperm between the Co and Tr samples. These results correlate with the observation that MES treatment only had a significant effect on abm sperm at two hours post thaw.

However, to damage the acrosome I would assume that it is likely that the plasma membrane was also damaged although this was not observed. The damage to the plasma membrane may not be significant, but it shows the same trend as the acrosomal membrane, and in this case and in my opinion, the statistically not significant difference may suddenly become meaningful. And, although not significant, strengthen the probability that MES did some damage to the plasma

membrane. The damage does, however, appear to be small enough to still allow the semen to be used for normal AI.

5.4 Acrosomal integrity

The acrosomal status of sperm cells must be preserved when semen is handled, diluted, exposed to, or treated with solutions. Irreversible damage can be caused to the sperm that can result in either cell death or capacitation-like changes (Hammerstedt *et al.,* 1990). In the present study a significant difference was found for the number of sperm with intact and reacting acrosomes between the Co and Tr samples as indicated in Figs 4.4 and 4.5 and Table 4.1. Capacitation-like changes of the acrosome were thus induced when the Tr samples were exposed to the MES solution. It can be concluded that even though MES treatment had no visible or detrimental effect on the plasma membrane, it did however have a detrimental effect on the acrosomal membranes of the spermatozoa. Capacitation–like changes occurred prematurely in the affected spermatozoa which would render them unable to undergo the normal physiological acrosome reaction. Affected sperm would thus not be able to penetrate the zona pellucida to fertilise an oocyte.

The semen pellets of the Tr samples were re-suspended with IVF TALP containing Ca^{2+}. TALP is known to induce capacitation, enabling sperm to undergo the acrosome reaction (Fraser, 1987; Sirivaidyapong *et al.,* 2000). According to Bavister and Yanagimachi (1977) the pyruvate contained in TALP stimulates the acrosome reaction. The significant difference found for the intact and reacting acrosomes between the Co and Tr samples might, at least partially, be associated with the pyruvate and Ca^{2+} contained in the IVF TALP. Further research would be necessary to confirm that the damage to the acrosome was caused by MES. It is somewhat surprising that the acrosome was damaged, although the sperm membrane that is covering the acrosome was not visibly damaged by the exposure to MES.

Whatever the reason for the acrosome reaction was, with 50% normal acrosomes after freezing and thawing the semen, there were still enough spermatozoa with normal acrosomes to allow the semen to be used for a normal AI program.

5.5 Longevity of sperm

This study described observations made only on frozen-thawed semen. The fact that these sperm cells did not come into contact with either the uterine or tubal fluids could be one of the cause for the significant difference found for longevity of the prm sperm over time for both the Co and Tr samples.

In several studies the authors reported that the dilution of bull epididymal sperm with an acidic medium suppressed the activation of sperm movement and that the action is reversible when the extra-cellular pH of the medium is raised (Acott & Carr, 1984; Carr *et al.,* 1985; Jones & Murdoch, 1996). These findings suggest that a low extra cellular pH may be able to suppress movement of sperm under experimental conditions. The pH of epididymal plasma ranges from 5.8 in the bull to 7.2 in the hamster (Carr *et al.,* 1985; Caflish & DuBose, 1990). In this study, frozen-thawed semen was exposed to MES with a pH of 5, but with the addition of PBS after 60 seconds, the pH was restored to physiological levels. We can thus assume that the low pH of the MES did not have a detrimental effect on the sperm motility over time. This statement is supported by the fact that MES had no detectable detrimental effect of the plasma membrane integrity as discussed under 5.3 in this chapter.

As expected a significant difference was found for the proportion of immotile sperm over time, suggesting that progressively motile sperm became immotile over time, which can be related to the inability of sperm to survive for a long period.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

MES caused damage to the sperm cells. The damage did however, appear to be small enough to still allow the semen to be used for normal AI. This technique thus holds considerable commercial potential for saving valuable cattle genetic material during a FMD outbreak as well as its potential use for moving genetic material derived from buffalo out of FMD endemic areas.

Despite some individual variation between bulls, the use of MES treatment in general appeared to have some effect on the survivability of frozen-thawed sperm cells. The effect of MES on motility of frozen-thawed bull semen was minimal, as abm sperm were only affected two hours after thawing. Although no effect on the plasma membranes were observed, it can be assumed that some damage did occur due to the fact that the acrosomal membranes were affected significantly. The detrimental effects observed would potentially prevent some sperm from being able to fertilize an oocyte. A significantly large proportion of sperm did however, survive the treatment. It might thus be possible to treat frozen-thawed semen with MES to remove the threat of the transfer of FMD to susceptible cows through insemination with semen from a carrier bull. It is probable that this treatment would also work in frozen-thawed buffalo semen.

6.2 Recommendations

This study established that MES treatment of frozen-thawed semen does not render it unfit for use. The following step would be to treat FMDV positive semen with MES.

This would attempt to establish if treatment with MES, with a low pH, will be effective in removing FMDV from potentially infective bull semen.

Furthermore, a case-control study could be done to examine whether the MES treated semen was effective for impregnation of cows or to fertilise oocytes *in vitro*. The precise effects of identical samples from the same batch of both frozen treated and untreated semen on fertility rates could then be studied.

This work could also be repeated using semen from culled buffalo bulls. This would substantiate the hypothesis that bull semen is a good model and that frozen thawed buffalo semen would also be minimally affected by the addition of MES.

CHAPTER 7

APPENDICES

APPENDIX 1

Data sheet for evaluation of frozen-thawed semen

Bull identification	Name			Breed					
Date of evaluation									
Motility	% Progressive			% Aberrant			% Immotile		
Field 1									
Field 2									
Field 3									
Field 4									
Field 5									
Field 6									
Field 7									
Field 8									
Field 9									
Field 10									
Average motility	%			%			%		
Plasma membrane integrity	Live sperm: %			Dead sperm: %					
Acrosome integrity	Acrosome intact /200			Acrosome reacting /200			Acrosome lost /200		
Longevity	**T_0**			**T_1**			**T_2**		
Field 1	%P	%A	%I	%P	%A	%I	%P	%A	%I
Field 2									
Field 3									
Field 4									
Field 5									
Field 6									
Field 7									
Field 8									
Field 9									
Field 10									
Average longevity	%	%	%	%	%	%	%	%	%

P= progressive

A = aberrant

I = immotile

T_0 = immediately after thawing

T_1 = 1 hour after thawing

T_2 = 2 hours after thawing

APPENDIX 2

Additional experimental results

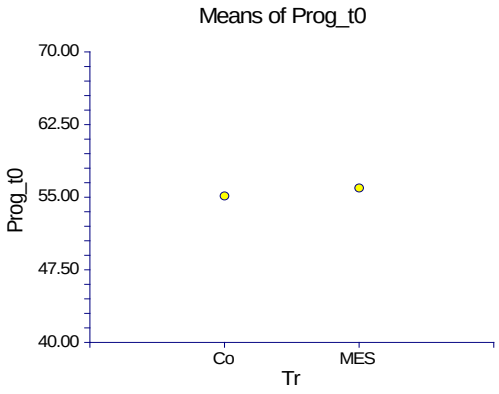

Fig 7.1: Influence of treatment on % progressively motile sperm immediately after thawing.

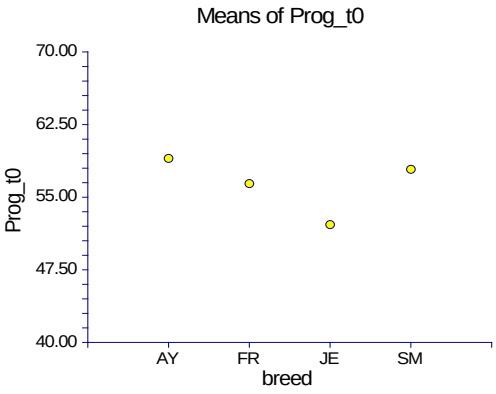

Fig 7.2 Influence of breed on % progressively motile sperm immediately after thawing.

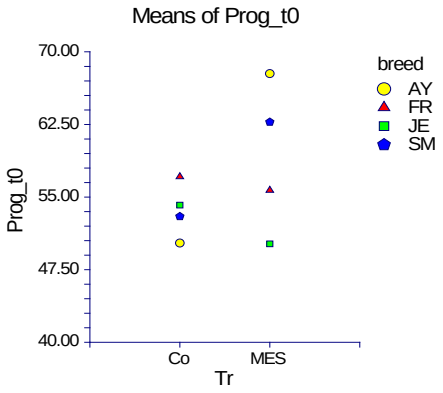

Fig 7.3: Influence of treatment and breed on % progressively motile sperm immediately after thawing.

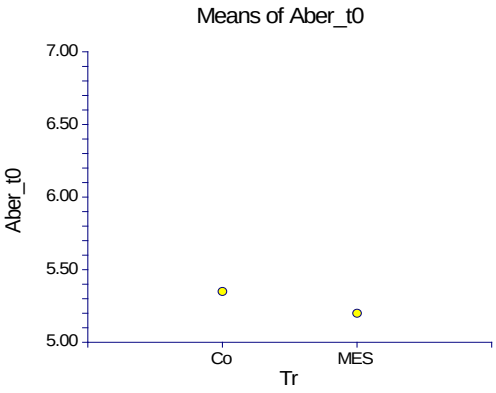

Fig 7.4: Influence of treatment on % aberrantly motile sperm immediately after thawing.

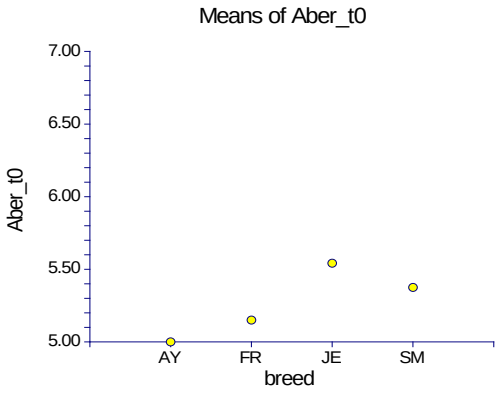

Fig 7.5: Influence of breed on % aberrantly motile sperm immediately after thawing.

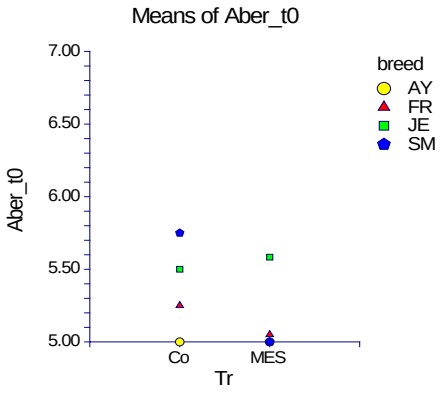

Fig 7.6: Influence of treatment and breed on % aberrantly motile sperm immediately after thawing.

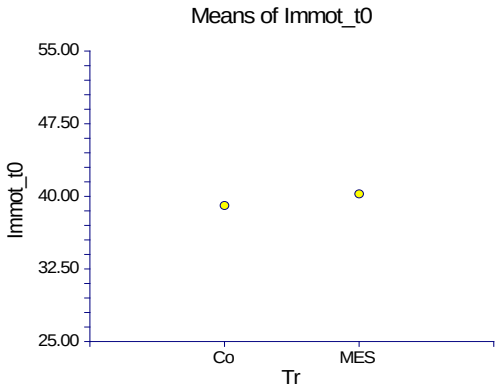

Fig 7.7: Influence of treatment on % immotile sperm immediately after thawing.

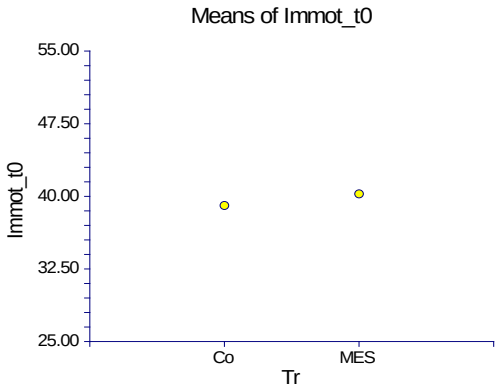

Fig 7.8: Influence of breed on % immotile sperm immediately after thawing.

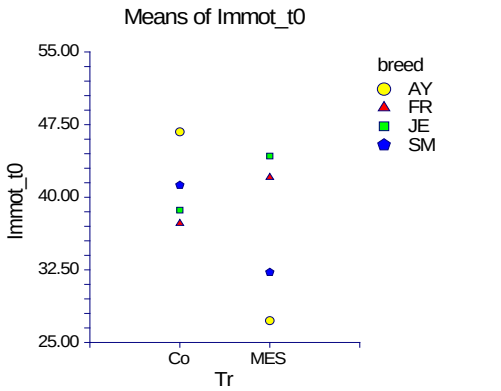

Fig 7.9: Influence of treatment and breed on % immotile sperm immediately after thawing.

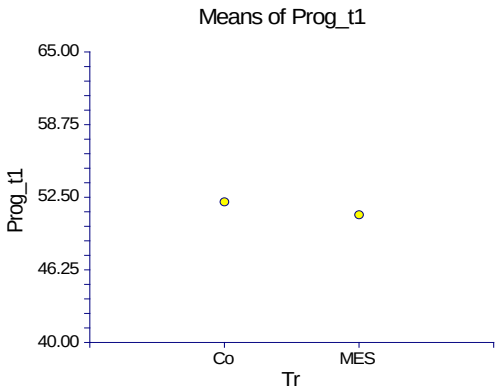

Fig 7.10: Influence of treatment on % progressively motile sperm 1 hour (t_1) after thawing.

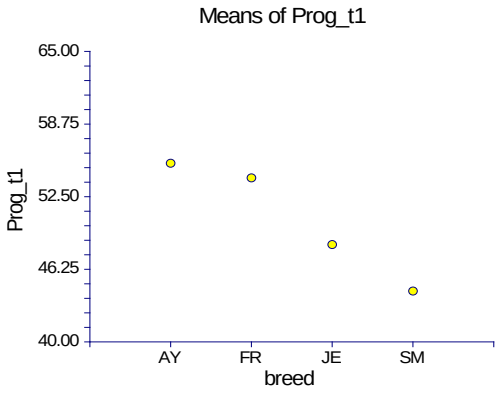

Fig 7.11: Influence of breed on % progressively motile sperm 1 hour (t_1) after thawing.

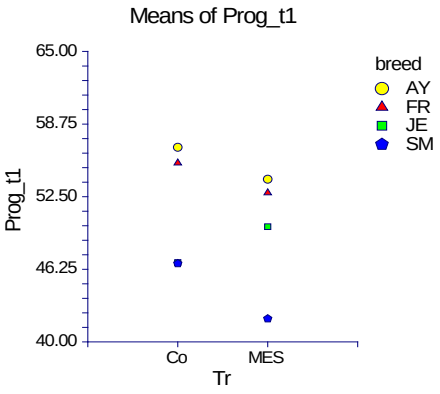

Fig 7.12: Influence of treatment and breed on progressively motile sperm 1 hour (t_1) after thawing.

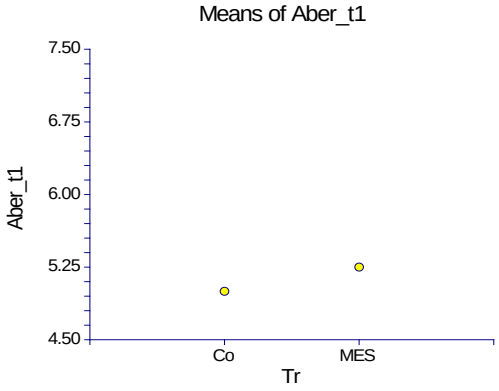

Fig 7.13: Influence of treatment on % aberrantly motile sperm 1 hour (t_1) after thawing.

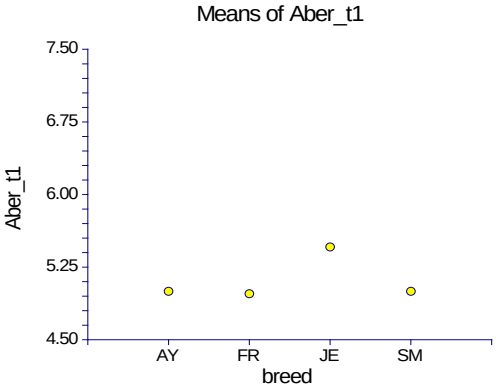

Fig 7.14: Influence of breed on % aberrantly motile sperm 1 hour (t_1) after thawing.

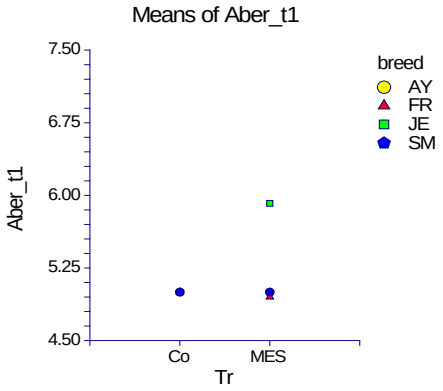

Fig 7.15: Influence of breed and treatment on % aberrantly motile sperm 1 hour (t_1) after thawing.

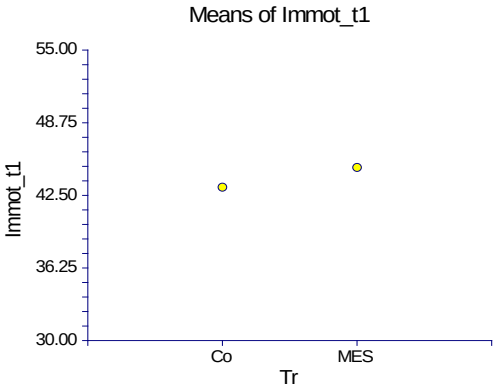

Fig 7.16: Influence of treatment on % immotile sperm 1 hour (t_1) after thawing.

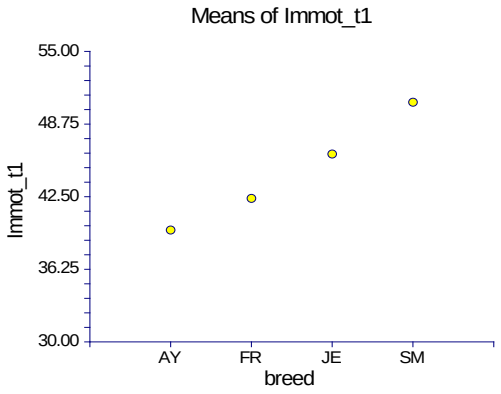

Fig 7.17: Influence of breed on % immotile sperm 1 hour (t_1) after thawing.

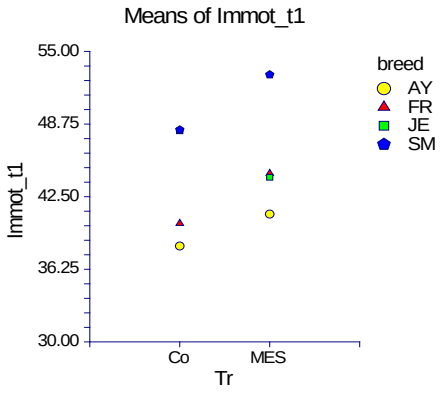

Fig 7.18: Influence of treatment and breed on % immotile sperm 1 hour (t_1) after thawing.

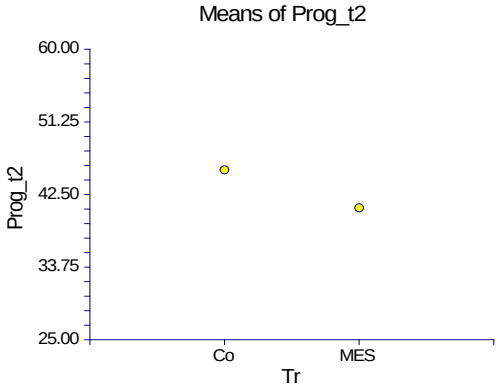

Fig 7.19: Influence of treatment on % progressively motile sperm 2 hours (t_2) after thawing.

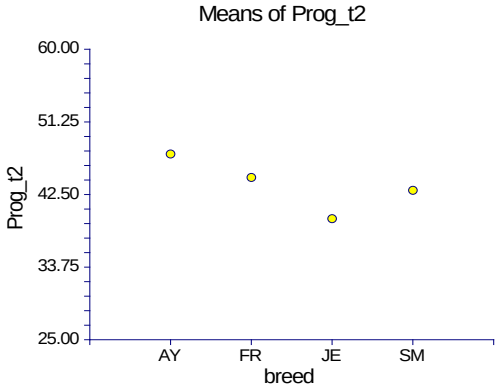

Fig 7.20: Influence of breed on % progressively motile sperm 2 hours (t_2) after thawing.

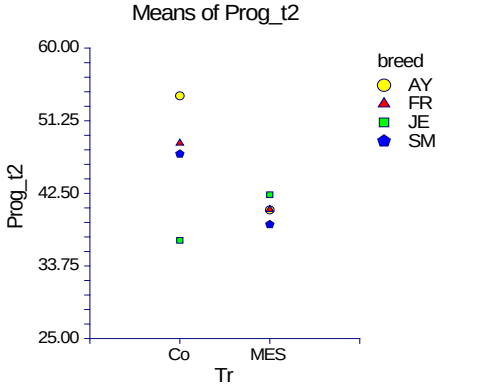

Fig 7.21: Influence of treatment and breed on % progressively motile sperm 2 hours (t_2) after thawing.

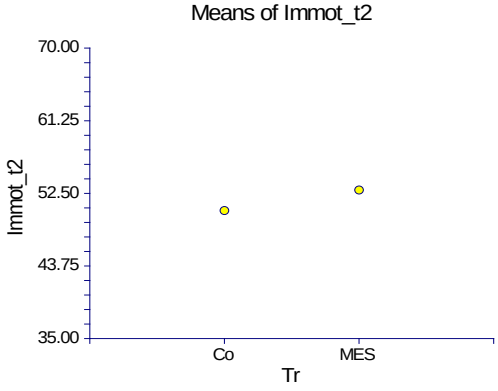

Fig 7.22: Influence of treatment on % immotile sperm 2 hours (t_2) after thawing.

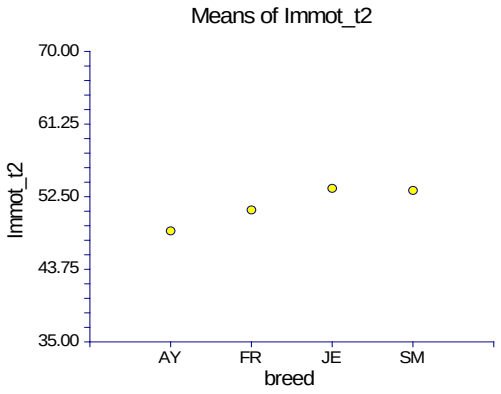

Fig 7.23: Influence of breed on % immotile sperm 2 hours (t_2) after thawing.

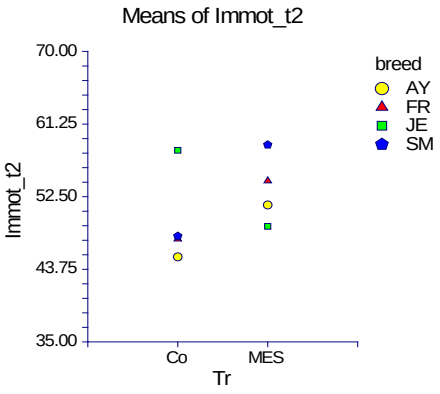

Fig 7.24: Influence of treatment and breed on % immotile sperm 2 hours (t_2) after thawing.

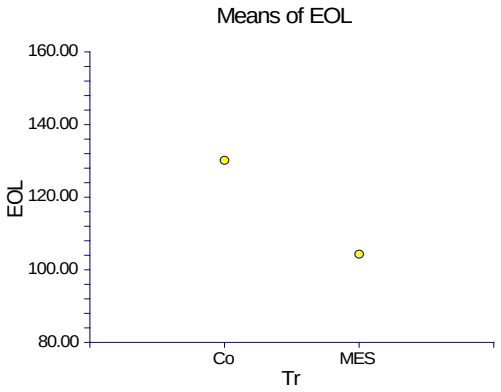

Fig 7.25: Influence of treatment on the number of sperm with intact plasma membranes.

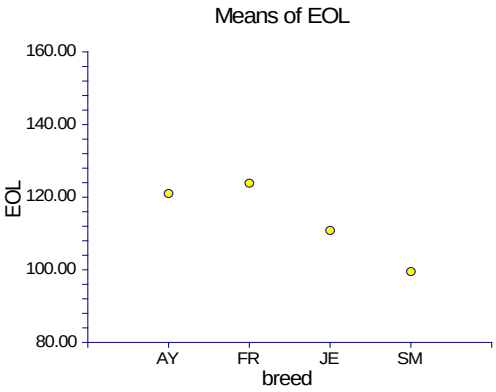

Fig 7.26: Influence of breed on the number of sperm with intact plasma membranes.

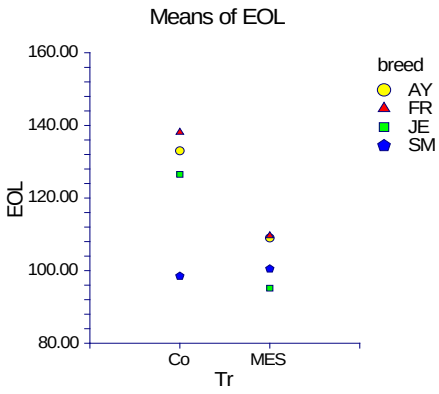

Fig 7.27: Influence of treatment and breed on the number of sperm with intact plasma membranes.

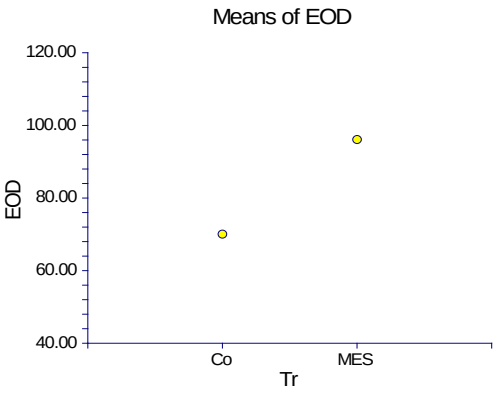

Fig 7.28: Influence of treatment on the number of sperm with damaged plasma membranes.

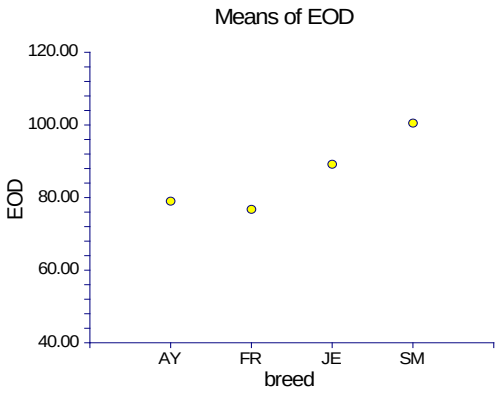

Fig 7.29: Influence of breed on the number of sperm with damaged plasma membranes.

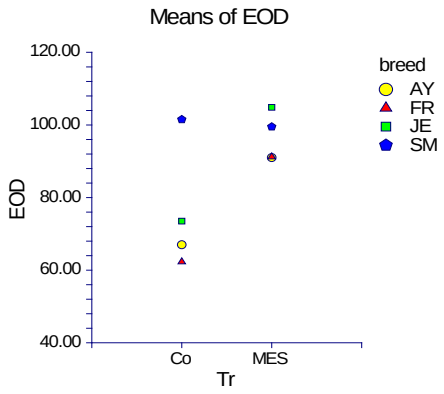

Fig 7.30: Influence of treatment and breed on the number of sperm with damaged plasma membranes.

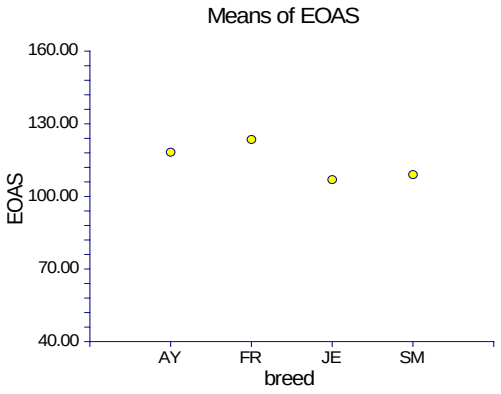

Fig 7.31: Influence of breed on the number of sperm with intact acrosomes.

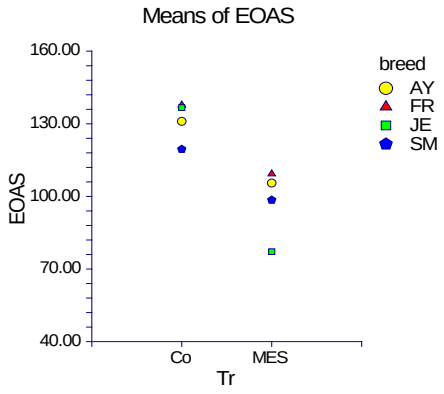

Fig 7.32: Influence of treatment and breed on the number of sperm with intact acrosomes.

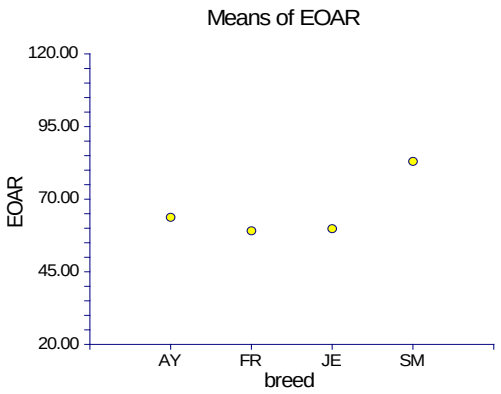

Fig 7.33: Influence of breed on the number of sperm with reacting acrosomes.

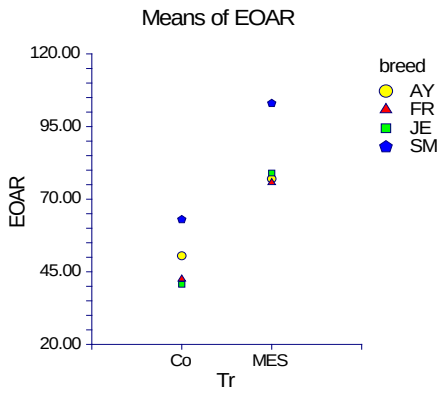

Fig 7.34: Influence of treatment and breed on the number of sperm with reacting acrosomes.

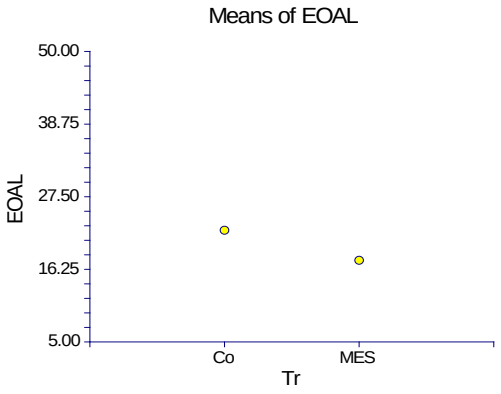

Fig 7.35: Influence of treatment on the number of sperm with lost acrosomes.

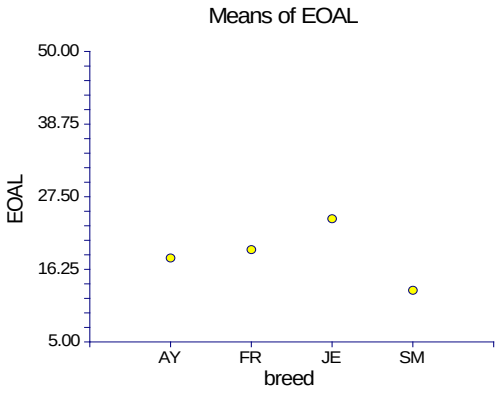

Fig 7.36: Influence of breed on the number of sperm with lost acrosomes.

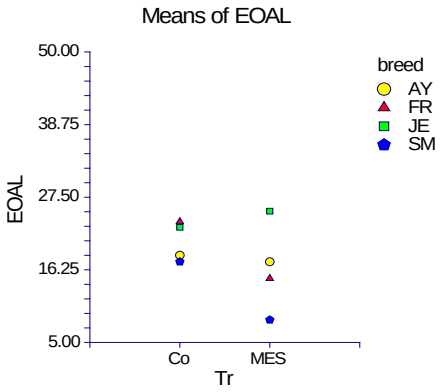

Fig 7.37: Influence of treatment and breed on the number of sperm with lost acrosomes.

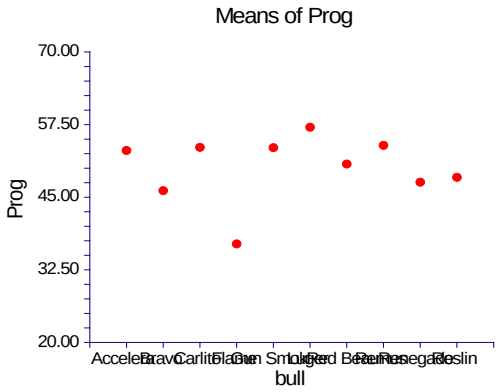

Fig 7.38: Influence of individual bulls on the longevity of the % progressively motile sperm.

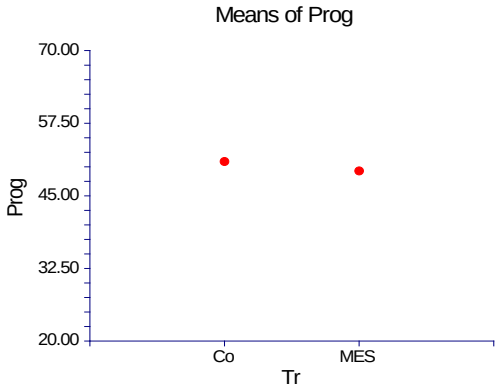

Fig 7.39: Influence of treatment on the longevity of the % progressively motile sperm.

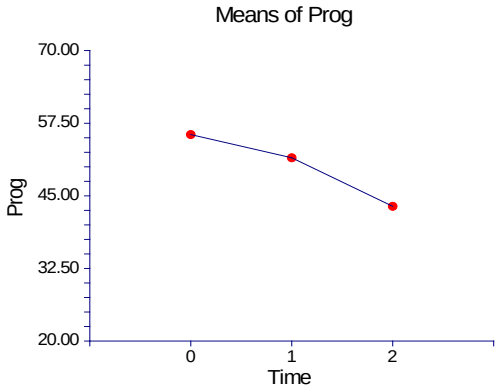

Fig 7.40: Influence of time on the longevity of the % progressively sperm.

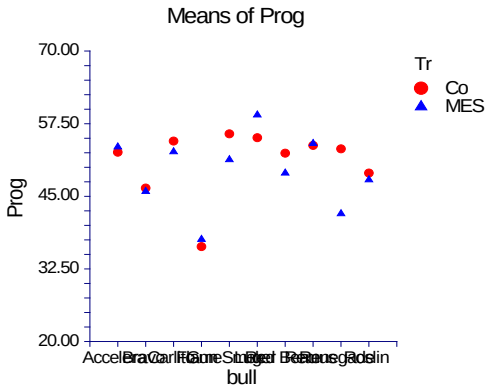

Fig 7.41: Influence of treatment on the longevity of the % progressively motile sperm of individual bulls.

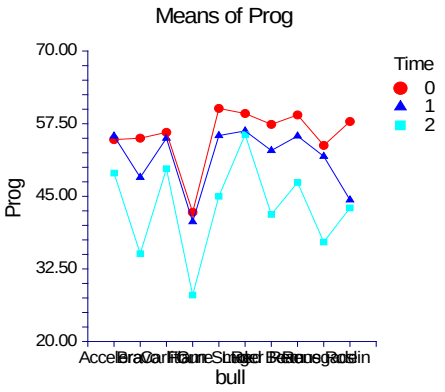

Fig 7.42: Influence of treatment and time on the longevity of the % progressively motile sperm of individual bulls.

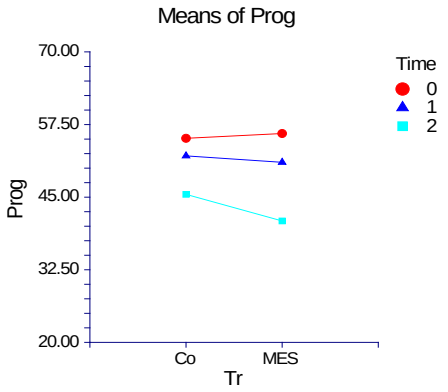

Fig 7.43: Influence of treatment and time on the longevity of the % progressively motile sperm.

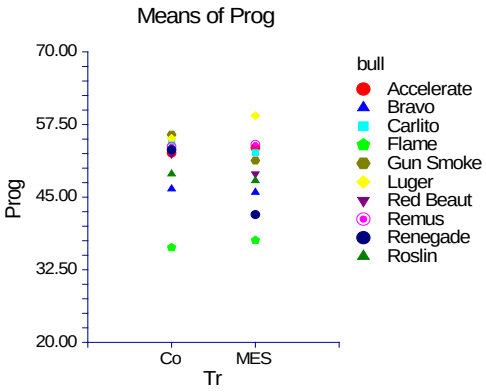

Fig 7.44: Influence of treatment on the longevity of the % progressively motile sperm of individual bulls.

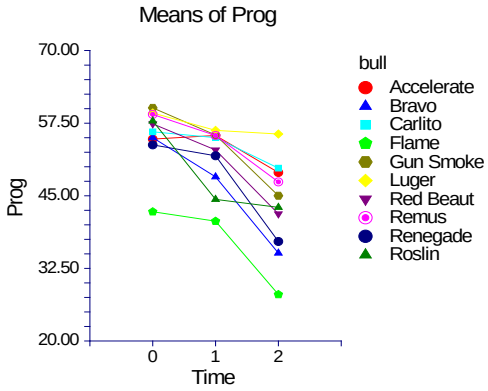

Fig 7.45: Influence of time on the longevity of the % progressively motile sperm of individual bulls.

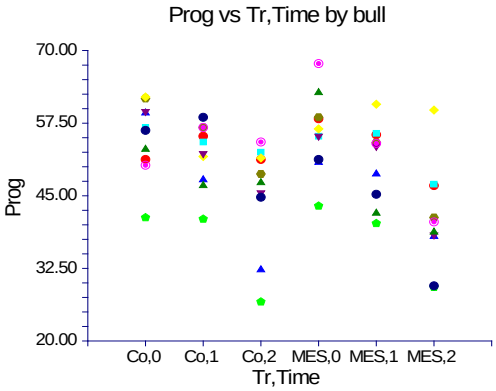

Fig 7.46: Influence of treatment and time on the longevity of the % progressively motile sperm of individual bulls.

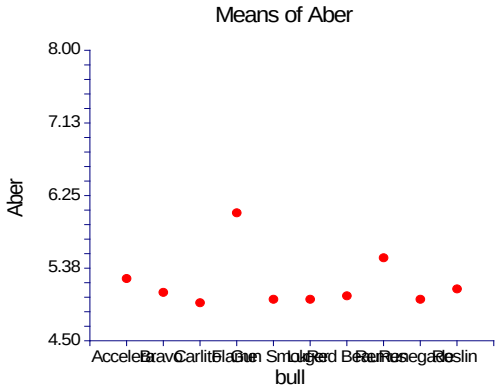

Fig 7.47: Influence of bull on the longevity of % aberrantly motile sperm.

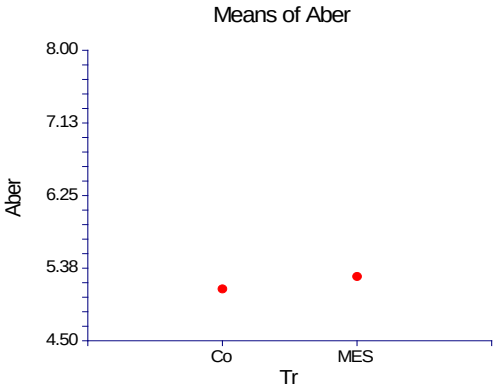

Fig 7.48: Influence of treatment on the longevity of the % aberrantly motile sperm.

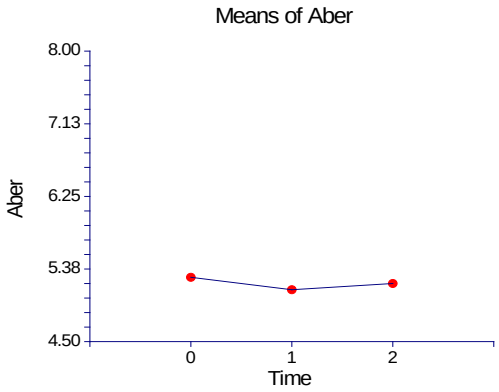

Fig 7.49: Influence of time on the longevity of the % aberrantly motile sperm.

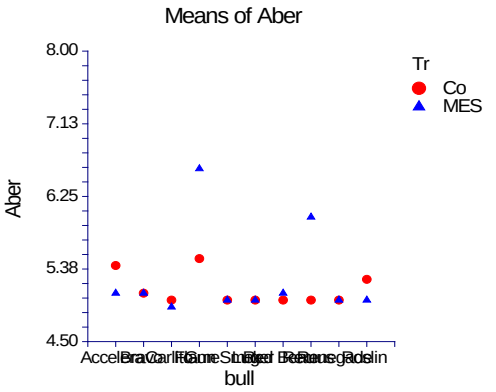

Fig 7.50: Influence of treatment on the longevity of % aberrantly motile sperm for individual bulls.

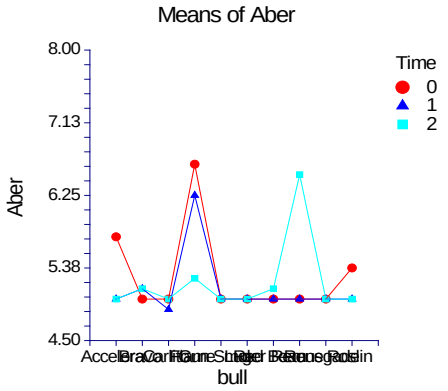

Fig 7.51: Influence of treatment and time on the longevity of % aberrantly motile sperm for the individual bulls.

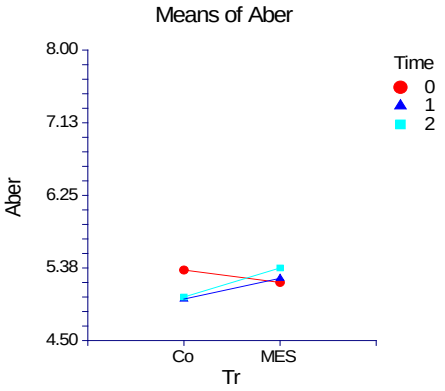

Fig 7.52: Influence of treatment and time on the longevity of the % aberrantly motile sperm.

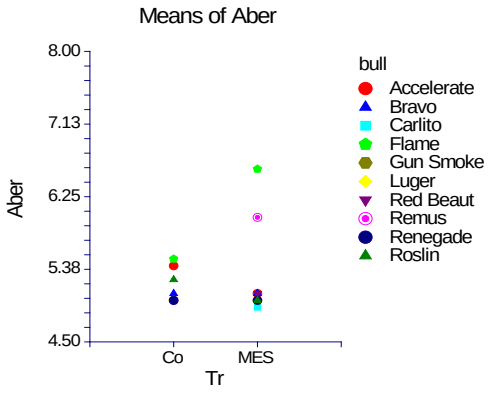

Fig 7.53: Influence of treatment on the longevity of the % aberrantly motile sperm for the individual bulls.

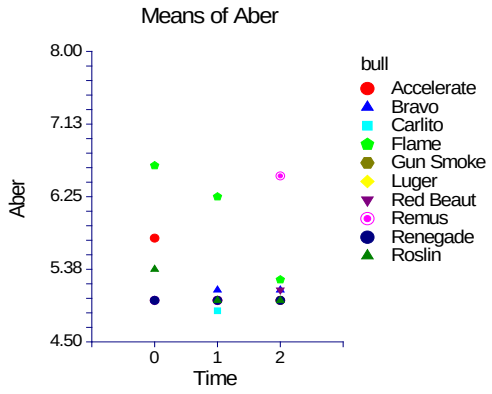

Fig 7.54: Influence of time on the longevity of the % aberrantly motile sperm for the individual bulls.

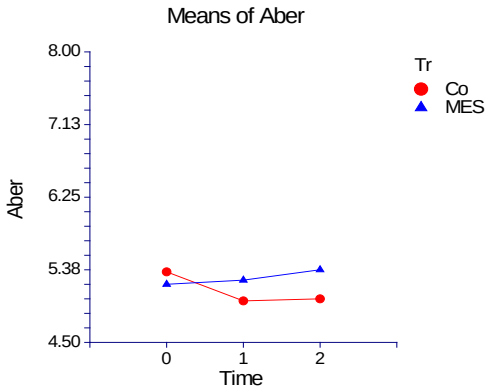

Fig 7.55: Influence of time and treatment on the longevity of the % aberrantly motile sperm.

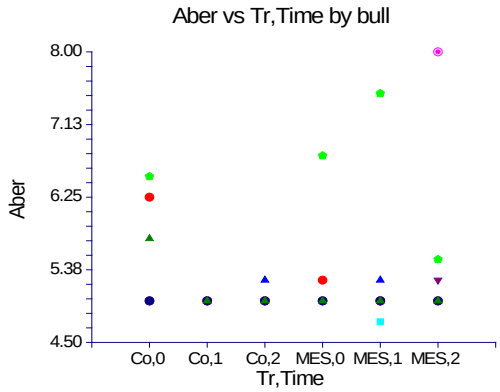

Fig 7.56: Influence of treatment and time on the longevity of the % aberrantly motile sperm for the individual bulls.

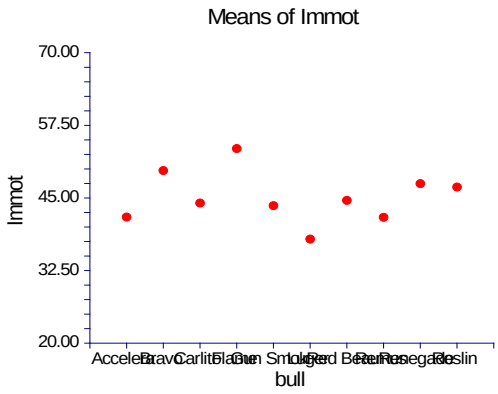

Fig 7.57: Influence of individual bulls on the % immotile sperm.

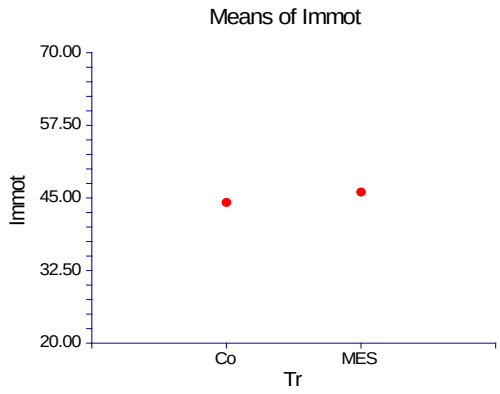

Fig 7.58: Influence of treatment on % immotile sperm.

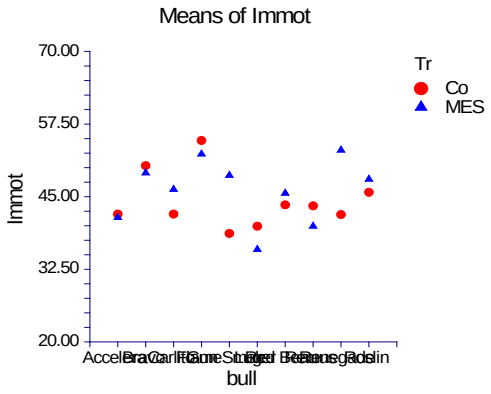

Fig 7.59: Influence of treatment on the % immotile sperm for the individual bulls.

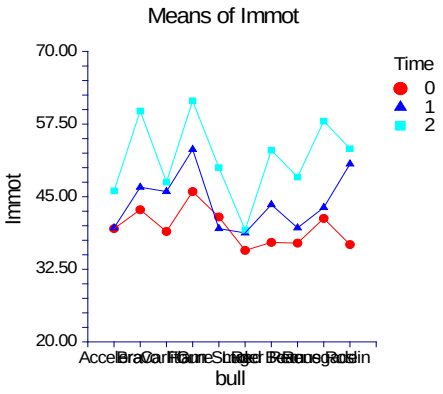

Fig 7.60: Influence of treatment and time on the % immotile sperm for the individual bulls.

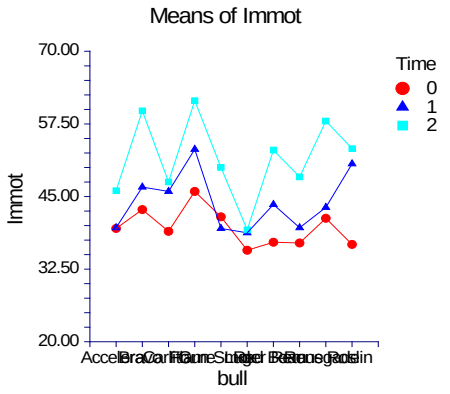

Fig 7.61: Influence of treatment and time on the % immotile sperm.

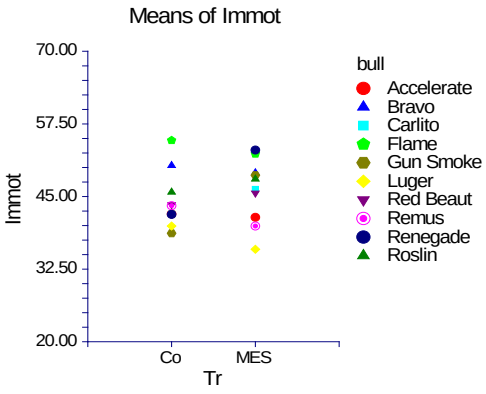

Fig 7.62: Influence of treatment on the % immotile sperm for the individual bulls.

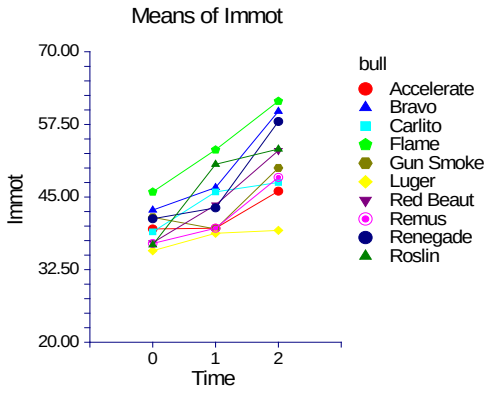

Fig 7.63: Influence of time on the % immotile sperm for the individual bulls.

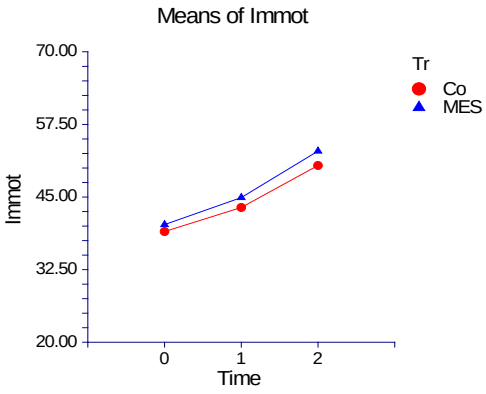

Fig 7.64: Influence of treatment and time on the % immotile sperm.

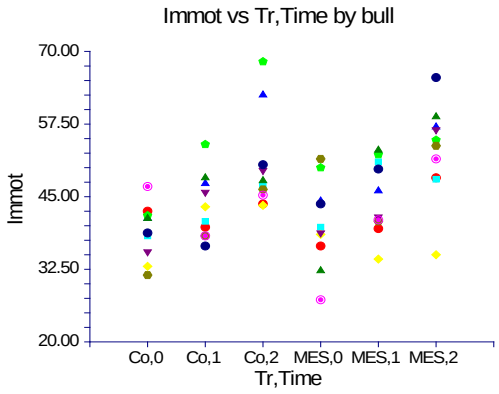

Fig 7.65: Influence of treatment and time on the % immotile sperm for the individual bulls.

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