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INFLUENCE OF EQUILIBRATION TIME AND FREEZING DILUENT ON POST-THAW MOTILITY AND ACROSOMAL INTEGRITY OF EPIDIDYMAL SPERM FROM THE AFRICAN BUFFALO (*SYNCERUS CAFFER*)

By

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Submitted in partial fulfilment of the requirements for the degree of Master of Science in the Department of Production Animal Science in the Faculty of Veterinary Science, University of Pretoria

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SUMMARY

INFLUENCE OF EQUILIBRATION TIME AND FREEZING DILUENT ON POST-THAW MOTILITY AND ACROSOMAL INTEGRITY OF EPIDIDYMAL SPERM FROM THE AFRICAN BUFFALO (*SYNCERUS CAFFER*)

By

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The aim of this study was to test whether or not the equilibration time of two different cryodiluents influences the post thaw qualities of epididymal African buffalo (*Syncerus caffer*) sperm. Diluents and equilibration times were compared by assessing the post thaw spermatozoal motility, longevity and the acrosomal integrity.

African buffaloes belong to Africa's "Big Five" and are, therefore, popular animals amongst game farmers, hunters and tourists. They are also asymptomatic carriers of foot-and-mouth-disease (FMD) and considered to be a wildlife reservoir for this plague. Other diseases, that are carried and can be transmitted from the African buffalo (*Syncerus caffer*) to livestock include tuberculosis, brucellosis and theileriosis or corridor disease (CD). Therefore, the transportation of African buffaloes is highly regulated. Disease-free buffalo populations are currently derived from a small genetic

pool and are smaller in their trophy size than the free-ranging animals from the diseased areas of the Kruger National Park (KNP) and the Hluhluwe/Umfolozi National Park. Hence there is a special interest in bringing new genetic material into the disease-free populations.

Epididymal sperm from 11 mature African buffalo bulls was collected, diluted with two different semen extenders (Triladyl™ [Tris egg yolk extender] and AndroMed® [synthetic extender, i.e. fully defined medium]) and frozen. Pre-freezing equilibration times of 2 and 9 hours were tested. Total and progressive motilities, longevities and acrosomal integrity were measured and compared.

Results show that there were no differences in post-thaw sperm quality when equilibration times between 2 and 9 hr were used. The use of the egg yolk containing extender (Triladly™) resulted in higher percentage of post-thaw motilities than the use of the synthetic AndroMed®.

Because a high percentage of progressive motile spermatozoa is one of the prerequisites for successful AI it must be concluded that Triladyl™ is superior to AndroMed®. As I believe the advantages of higher motility to be bigger than the hygiene risks of a yolk containing extender I conclude that epididymal buffalo sperm should rather be frozen with Triladyl™ than with AndroMed®.

Keywords:

African buffalo, epididymal sperm, equilibration time, egg yolk free, AndroMed, Triladyl, cryopreservation, FITC-PNA stain, longevity, buffaloes free of specific diseases

Opsomming

DIE INVLOED VAN EKWILIBRASIETYD EN BEVRIESINGSVERDUNNER OP DIE BEWEEGLIKHEID EN AKROSOMALE INTEGRITEIT NA ONTDOOIING VAN EPIDIDIMALE SPERMS VAN DIE AFRIKAANSE BUFFEL (*SYNCERUS CAFFER*)

Deur

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Graad: MSc

Die doel van hierdie studie was om vas te stel of die eienskappe van epididimale sperms van die Afrikaanse buffel (*Syncerus caffer*) na ontdooiing beïnvloed word deur die ekwilibrasietyd en die twee verdunners waarin dit bevries is. Verdunners en ekwilibrasietye is vergelyk met betrekking tot die beweeglikheid, langlewendheid en akrosomale integriteit van die sperms na ontdooiing.

Afrikaanse buffels behoort tot Afrika se "Groot vyf" en is daarom gewild onder wildboere, jagters en toeriste. Hulle is simptoomvrye draers van bek-en-klouseer en word daarom beskou as die bron van hierdie plaag onder wilde diere. Afrikaanse buffels dra ook tuberkulose, brucellose en buffelsiekte na vee oor. Afrikaanse buffels mag gevolglik nie sonder 'n spesiale vervoerpermit verskuif word nie. Die genepoel van siektevrye buffels is klein. Boonop is die trofeë van siektevrye buffels kleiner as dié van diere uit besmette gebiede soos die Nasionale Kruger Wildtuin en die

1 LITERATURE REVIEW

1.1 The African buffalo (*Syncerus caffer*)

The African buffalo (*Syncerus caffer*) is one of the wildlife species that suffered most from the colonization of Africa by Europeans. This was due to the importation of foreign diseases such as Rinderpest (De Vos 1987). After the outbreak at the end of the 19th century only a few relic populations remained in the Kruger National Park, the Hluhluwe and Umfolozi Nature Reserves and the Addo Elephant National Park.

Although there is a great awareness of the importance of preserving our natural heritage and maintaining the diversity of animals and plants throughout the world's population, one can never be certain that a situation involving low numbers of individuals will not come to be in the future.

The Daily News (Tanzania), for example, reported a decrease in the total number of African buffaloes in the Serengeti of about ten percent per year due to poaching between 1986 and 1992. The situation in the Tarangire National park is even worse; the decline was about 30% in 1996. In Mocambique massive eradication of buffalo herds occurred over the last two decades for the provision of meat during times of war (Prins 1996).

Fortunately the African buffalo is not considered as an endangered species within the Republic of South Africa and the numbers are very favourable. However, as the African buffalo belongs to Africa's "Big Five" the demand for so-called "disease free" animals in the private sector for breeding purposes and trophy hunting exceeds the availability at the moment (De Vos 1987).

There are restrictions on the transport of living cloven-hoofed animals due to the fact that the Republic of South Africa is only partly foot-and mouth-disease (FMD) free. Large numbers of African buffaloes occur in FMD endemic areas such as the Kruger National Park. The African buffalo is believed to be the only long term carrier and reservoir for SAT viruses, which are the causative agents of FMD in South Africa (Bengis *et al.* 1987, Hedger and Condy 1985).

Transmission of FMD between buffaloes and cattle is not a common event (Anderson 1986, Bengis *et al.* 1987) and under normal field conditions only buffaloes in an acute stage of the infection are likely to infect domestic cattle, provided that close contact occurs (Gainaru *et al.* 1986). A study described by Bastos *et al.* (1999a) shows that SAT-type FMDV can be present in ejaculated semen of African buffaloes even if they do not show any clinical signs of infection. Virus isolation was however never described from epididymal sperm. Permit control for movement of buffaloes and buffalo products has been established to prevent spreading of FMD (Bengis *et al.* 1986).

Another important aspect regarding breeding, specifically related to transport and translocation of African buffaloes is that they are the only ruminants that are carriers for Corridor disease, caused by *Theileria parva lawrencia* (Potgieter *et al.* 1988). This disease can also be transmitted to domestic animals and cases have been reported in the 1960s and again in the early 1980s in cattle herds adjacent to the KNP. These incidences give indications that close contact between cattle and buffaloes took place.

Bovine tuberculosis is caused by *Mycobacterium bovis* and is an exotic disease in Southern Africa. It was first brought into the region by English and Dutch cattle during colonization in the 19th century (Tanner and Michel 1999). The first time the disease was detected in South Africa in African buffaloes was in 1990 in the Kruger National Park (KNP). Since outbreaks of tuberculosis in above mentioned cattle herds were reported at the same time as the Corridor Disease outbreak, it is likely that this was the time when tuberculosis was first brought into the National Park (Bengis *et al.* 1996). Epidemiological surveys on bovine tuberculosis in buffaloes have been conducted since 1993 in the Hluhluwe/Umfolozi National Park. Prevalences exceeded 70% in the worst affected herds (personal communication Jolles, A. Department of Ecology and Evolutionary Biology; Princeton University, USA). Although never described in African buffaloes, genital tuberculosis occurs in cattle (Rosenberger 1978).

Brucella abortus was first isolated in South Africa in 1977 from African buffaloes in the Kruger National Park (Gradwell *et al.* 1977). It is suggested in this study that African buffaloes are not the original reservoir of brucellosis, but rather have obtained

the disease from cattle. They are nevertheless considered to be a source of re-infection for domestic stock (Herr and Marshall 1981). This disease, which can induce abortion and orchitis, is transmitted through aborted foetuses, fetal membranes, placental fluids, placenta and semen (Rosenberger 1978). A case of orchitis in buffaloes has been described in Tanzania (Kaliner and Staak 1973). Even though serologically positive animals were found frequently, the isolation of *Brucella abortus* has proven to be difficult. In this report out of 7 animals that showed agglutination titres only one was bacteriological positive and showed clinical signs. These were similar to a unilateral *Brucella*-orchitis seen in cattle and it was concluded that the infection was chronic and lead to sterility. Another study reports that despite of 28% positive animals found in a herd, there was still a high number of pregnant or lactating cows. Therefore and because of the rate of increase in numbers of buffaloes in the KNP it is suggested that Brucellosis hardly affects the reproduction in this species (De Vos and van Niekerk 1969).

As the animals of the Kruger National Park and the Umfolozi/Hluhluwe National Park complex originate from a large genetic pool and are generally larger than buffaloes found elsewhere in the country there is a special desire to reap the benefits of these genetic pools (Gerber 2000). Assisted reproductive techniques might prove to be an important tool to bypass the difficulties regarding the transport of African buffaloes whilst still benefiting from the genetic pool of diseased areas. Another advantage of artificial insemination and in-vitro fertilisation would be that samples taken from buffaloes can be tested for diseases (especially those mentioned above). As also stated above, SAT-virus can also be present in semen of African buffaloes showing no clinical signs of infection (Bastos *et al.* 1999b). Studies in HIV-1 and Hepatitis C Virus (HCV) positive men indicate that the virus load in semen can be reduced to undetectable levels by washing and density gradient or swim-up separation of the semen (Hanabusa *et al.* 2000, Pasquier *et al.* 2000). The development of similar methods for African buffaloes may potentially decrease the risk of disease transmission if processed semen can be used for assisted reproductive technology.

1.2 Importance of cryopreservation of semen from the African buffalo

Cryopreservation not only provides an efficient and practical way for artificial breeding of domestic animals and wildlife but will also play a major role in preserving earth´s bio-and genetic diversity (Wildt 1992). This reproductive technique will open up the possibility of establishing a genetic bank, and prevents a loss in genetic diversity and a "bottleneck situation" after future catastrophes. Frozen germ cells not only extend the generation interval almost indefinitely, but also provide high levels of insurance against the loss of diversity or entire species (Wildt 1992).

The special importance of cryopreservation of semen from the African buffalo arises from the large demand for animals with large trophies by private game farms, and the restriction on transport of African buffaloes.

Due to the above mentioned matters different attempts have been made to produce buffaloes free of specific diseases (so called disease free buffalo) according to the demands of private game reserves, of which cryopreservation is one.

Attempts to freeze epididymal sperm from African buffaloes have been made in the past. Cryodiluents used were sperm-Tyrodes's-albumin-lactat-pyruvate (sperm-TALP) (Bartels *et al.* 1996; Lambrechts *et al.* 1999), Biladyl™ (Minitüb, Germany) (Kilian *et al.* 2000; Lubbe *et al.* 2000), Triladyl™ (Minitüb, Germany) (Bartels *et al.* 1996; Gerber *et al.* 2001; Lambrechts *et al.* 1999 and Lubbe *et al.* 2000) and a mixture of Hams F-10 (with sodium bicarbonate but without glutamine) and Triladyl™ (Minitüb, Germany) (Killian *et al.* 1993). The means of total post-thaw motilities achieved in these studies have ranged between 19% and 45%.

There always remain questions about the ideal method and the need for further studies to develop a specialized cryopreservation protocol has been stated.

1.3 Anatomy and histology of the epididymis in bulls (*Bos taurus*)

The epididymis is closely attached to the testis and connected by the meso-epididymis. The epididymis can be divided into three different parts: the head (caput epididymidis), the body (corpus epididymidis) and the tail (cauda epididymidis). The

epididymal duct forms lobules which are partially isolated by septa of connective tissue.

The epithelium is formed by pseudostratified cells, which occur in a single layer. Five different cell types can be found, these are: principal, apical, clear, basal and halo cells.

The efferent ducts and epididymal canal are surrounded by a circular layer of smooth muscle cells, which receive their noradrenergic innervation from ganglionic neurones that receive hypogastric and pelvic afferents. The epididymis also receives cholinergic and peptidergic innervations.

Caput and corpus are vascularised by two arteries originating from the spermatic arteries; a branch of the iliac artery irrigates the vas deferens and the cauda epididymides (Fournier-Delpech and Thibault 1993).

1.4 Physiology

1.4.1 Transport, sperm maturation and storage in the epididymis

The seminiferous epithelium releases immotile spermatozoa continuously; these are carried through the ductuli efferentes and the initial epididymal segment. The transport within the epididymis itself is mainly dependent on rhythmic contractions (every 6-20 seconds) of the surrounding smooth musculature. The duration of transport in the bull is about two days each for the caput and the corpus, and does not vary with ejaculation frequency (Fournier-Delpech and Thibault 1993).

Spermatozoa undergo transformations within the epididymis in order to gain the ability of linear forward motility as well as the ability to fertilize and initiate embryonic development. These characteristics are acquired continuously and independently (Fournier-Delpech and Thibault 1993). Although various changes have been described their functions are not yet understood. Suzuki (1981) describes rearrangements in the plasma membrane which occur in different species and are, at least in the boar, not restricted to a single region and take place after the migration of the cytoplasmic droplet. He suggests that these alterations give added rigidity to the membrane structure during spermatozoal transport through the male and female

genital tract and suggests in a more recent paper (Suzuki 1988) that cholesterol is essential for membrane stability and permeability.

Proteins are secreted by the epididymis (Kohane *et al.* 1980a; Kohane *et al.* 1980b; Vreeburg *et al.* 1992) which bind to the spermatozoa. Some of these proteins stabilize the plasma membrane and may prevent premature acrosome reaction (Reynolds *et al.* 1989; Thomas *et al.* 1984).

However Fournier-Delpech and Thibault (1993) describe that spermatozoa gain the ability to bind to the zona pellucida during their passage through the caput epididymidis. This ability is dependent upon membrane proteins of testicular origin, which act as proreceptors, and only become functional after androgen dependent proteins secreted in the epididymal fluid, are deposited on the sperm plasma membrane. The development of the sperm´s ability to move properly seems to be attributed to a forward motility protein (FMP) - which is found in its highest concentration in the cauda epididymidis (Brandt *et al.* 1978), the epididymal fluid, and the development of a system that keeps the intracellular calcium low (Vijayraghavan and Hoskins 1990).

The storage of the spermatozoa occurs in the cauda, where they can survive more than 3 weeks. The sperm content here, in contrast to the above-mentioned head and body, is dependent on both sexual activity and contractions of smooth muscle, which are stimulated by oxytocin and vasopressin, released during coitus (Fournier-Delpech and Thibault 1993).

Even though stored spermatozoa are fully capable of forward movement they are kept in a quiescent state. It has been found that spermatozoa in vitro stay immotile in cauda epididymal fluid (CEF) but dilution results in initiation of motility (Turner and Howards 1978). The same study also proves that quiescence is enforced by epididymal plasma rather than cell-to-cell contact, since dilution with sperm free cauda luminal plasma did not result in sperm motility. It is furthermore stated here that neither alteration of osmolality, nor Calcium, nor any particular ion, nor addition of oxygen stimulates motility in neat epididymal fluid.

After experimenting with rats it has been suggested that a proteinaceous factor is responsible for maintaining epididymal sperm immotile. This factor is said to be present in more proximal parts of the epididymis as well, but becomes either more active or increases in concentration further distally (Turner and Giles 1982).

It has also been found that glycerolphosphocholin (GPC) and carnitin, which are both present in high concentrations in rat CEF, are capable of suppressing sperm motility in vitro (Turner *et al.* 1978).

When different species are compared it becomes apparent that CEF of one species can immobilize spermatozoa from another. Guinea pig spermatozoa for example become immotile in rat CEF. Rabbit spermatozoa have been found to be motile in the cauda epididymidis. This is the only exception amongst the five investigated species (rat, hamster, guinea pig, human). The former existing opinion of viscoelasticity being a major factor in the quiescence of rat spermatozoa has been contradicted in this study, since CEF fluids of other species with low viscoelasticity also inhibited the sperm motility (Turner and Reich 1985).

Bull spermatozoa are also potentially motile but stored in a quiescent state. It has also been suggested that a motility inhibiting factor is present in bovine CEF (Carr and Acott 1984). This factor has been found to be dependent on pH and to get inactivated by increasing the pH, which happens naturally upon dilution of CEF with seminal plasma (Acott and Carr 1984).

1.4.2 Composition of seminal plasma

"The seminal plasma, an extracellular fluid which provides the medium and vehicle for spermatozoa, is a composite mixture of secretions which come from the male accessory organs of reproduction" (quoted from Mann 1964).

The seminal plasma does not only consist of cell secretions but also of cellular debris and exfoliated epithelial cells. This is a result of changes in the epithelial structure reaching from desquamation to cell rupture.

In bovines the seminal plasma is produced by the prostate gland, the seminal vesicle, the ampulla of the deferent duct, the bulbourethral gland and the urethral glands.

The prostatic fluid, a colourless secretion, is rich in citric acid, acid phosphatase and zinc. The prostate is also the main source of enzymes such as amine oxidase, transaminase as well as lactic, malic and isoitric dehydrogenase.

The secretion of the seminal vesicles, which should be correctly termed seminal glands in bovines, makes up a substantial portion of the whole ejaculate in bulls. It is usually slightly yellowish, which is due to its flavin content. It contains more potassium, bicarbonate, phosphate and protein than the prostatic fluid. The proteins in bull seminal plasma consists mainly of globulins and not albumins. One of the most characteristic chemical properties of the secretion of the seminal glands is its reducing power. This can be attributed to the presence of ergothioneine, ascorbic acid and sugar reducing substances such as cupric hydroxide. The seminal glands also produce the bulk of fructose contained in seminal plasma. This is especially true for the bull and the ram which are both efficient fructose producers.

The ampullary glands are glandular enlargements of the terminal portion of the ductus deferentia. Its secretion is also a yellowish-coloured fluid, rich in ergothioneine and phosphor. It also contains fructose in bulls, in contrast to stallions, whose ampullary glands however exceed the size of the other accessory glands. The ampullary fluid contains also the same amino acids as the vesicular fluid, namely serine, glycine, alanine, aspartic acid and glutamic acid.

The bulbourethral glands are a pair of small bean shaped organs in the bull and are not as important as in the boar. Situated close by are the urethral glands which void their colourless, viscous secretion, which is rich in mucopolysacharides and chloride, in the urethra along its entire length (Mann 1964).

1.4.3 Functioning of seminal plasma

1.4.3.1 Coating

During process of ejaculation epididymal sperm gets in contact with seminal plasma and proteins contained in it. The binding of such proteins, named spermadhesins to the acrosome is termed coating. A protein has been found in bulls that is not present in epididymal sperm but becomes coated on ejaculated sperm. It has been named acidic seminal fluid protein (aSFP). Its presence decreased during incubation in

capacitation medium. This lead the authors to the suggestion that aSFP plays a role as a decapacitation factor (Dostalova *et al.* 1994a). This spermadhesin is synthesized by the ampulla and seminal vesicle epithelium in bulls and its functional properties on molecular basis have recently been established (Einspanier *et al.* 1994).

Other studies also show that seminal plasma-treated epididymal bull sperm was unable to undergo acrosome reaction unless exposed to capacitation factors. These results confirm the prior mentioned ones in that the coating of sperm cells during ejaculation inhibits premature capacitation (Flormann and First 1988).

Similar studies performed in boars showed that of the spermadhesin protein family only AWN-1 is present on epididymal sperm. AQN-1, AQN-2, AQN-3 and AWN-2 become coated during contact of spermatozoa with seminal plasma. Similar to the results in boars these proteins were released from the sperm's head during an incubation period of 3 h of in vitro capacitation. It is therefore also concluded in this article that these spermadhesins act as decapacitation or acrosomal stabilizing factors (Dostalova *et al.* 1994b).

In addition to aSFP another spermadhesin has been isolated from bull seminal plasma and named Z 13. It possesses similar qualities as aSFP and the two distinguish themselves from the spermadhesins found in boars in that they are not heparin binding (Tedeschi *et al.* 2000).

1.4.3.2 Influence on motility

The effect of seminal plasma on the motility of fresh epididymal spermatozoa is described as detrimental in boars (Berger and Clegg 1985). In rams a favourable effect of seminal plasma on the post-thaw motility of epididymal sperm has been described (Graham 1994). In contrast, an improved spermatozoal motility of epididymal sperm in stallions has been found after addition of seminal plasma only before freezing but an adverse effect after thawing (Braun *et al.* 1994). There was however no effect found on the motility of epididymal bull spermatozoa after the addition of seminal plasma (Graham 1994).

The motility of bovine sperm cells that became immotile after washing with Ficoll and ressuspension with buffer could be restored by the addition of seminal plasma. It could also be seen that the lifespan of these spermatozoa was the shorter the more seminal plasma was added (Baas *et al.* 1983).

1.4.3.3 Influence on droplet

Early reports describe that seminal vesicular fluid has a removing effect on cytoplasmic droplets still attached to the sperm´s tail (Bialy and Smith 1958). This function was additionally studied more recently and a haemolytic factor, called phospholipid-binding-protein (PBP) was proven to be the causative agent for the release of the droplet (Matousek and Kysilka 1980).

1.4.3.4 Suppression of immune response

Another effect of seminal plasma is a suppression of the immune response from the female reproductive tract, which may protect the spermatozoa from being prematurely eliminated. An inhibition of bovine neutrophil phagocytosis by seminal plasma has been described (Strzemienski 1989), whereas other reports attribute immunosuppressive properties to the phospholipid binding protein (PBP) (Matousek and Stanek 1993). In addition to that it has been found that the sperm-motility-inhibiting-factor (SMIF) also affects the cell-mediated and humoral immune response, at least in boars (Velev *et al.* 1992).

1.4.3.5 Calcium and glucose uptake

Intracellular Calcium is a necessary factor in the regulation of contractile and secretory functions in many different cells including sperm cells. It has been shown that motility of bovine epididymal sperm cells was stimulated when Calcium influx could occur (Babcock *et al.* 1976).

Babcock *et al.* (1979) also relate the absorption of Calcium to motility initiation and describe that seminal plasma contains a factor which prevents or delays the uptake of Calcium into sperm mitochondria and that this factor may act upon the surface membranes of the sperm.

In another study it was found that calcium uptake also correlates with the acrosome reaction. It was suggested that Calcium is first bound to the outer acrosomal or plasma membrane and later released during acrosome reaction. What has been proven was that the acrosome reaction is dependant on a critical concentration of exogenous calcium (Singh *et al.* 1978).

A reduced net uptake of glucose has been described in bovine semen in the presence of seminal plasma. Three different reasons suggested were (A) that seminal plasma contains a substance, which is used in preference over glucose, (B) that seminal plasma inhibits the uptake of calcium or (C) that it stimulates glycolysis and the release of metabolites (Flipse 1954).

1.4.3.6 Capacitation and acrosome reaction

The accessory sex glands also produce heparin binding proteins (HPBs) and secrete them into the seminal plasma. The major source of HPBs in the bull are the seminal vesicles. Once HPBs are bound to the surface of sperm cells their ability to respond to capacitation-inducing glycosaminoglycans, such as heparin, increases (Nass *et al.* 1990).

Once spermatozoa are capacitated they are capable to undergo the acrosome reaction which is triggered by zona pellucida proteins. Experiments show that addition of seminal plasma to epididymal sperm increases the susceptibility of spermatozoa to these proteins (Flormann and First 1988). Sperm cells treated in such a way are therefore able to undergo the acrosome reaction, even when they are exposed to lower concentrations of zona pellucida proteins.

1.4.3.7 Fertility

Various studies in humans prove the relationship between the content of Glycerolphosphorylcholin (Jeyendran *et al.* 1989); Prostaglandins (Cosentino *et al.* 1984) and a so called P-factor (Gaur and Talwar 1975) in the seminal plasma and the fertilizing ability of spermatozoa. Similar qualities have been attributed to proteins found in the seminal plasma of Holstein Bulls (Killian *et al.* 1993).

1.4.4 Influence of seminal plasma on survivability and freezability

The survivability of spermatozoa at 37°C was found to be significant higher for Nili-Ravi buffalo bull sperm samples deprived of seminal plasma than it was for untreated semen (Ahmad *et al.* 1996). In other experiments the percentage of live as well as of motile spermatozoa has been increased by using a semen extender with a content of 10% seminal plasma in rams, boars and bulls (Maxwell *et al.* 1996). This study also showed that high levels of seminal plasma are toxic for porcine sperm cells.

A factor reducing the liveability of spermatozoa, which is suggested to be a protein has been found in seminal plasma. This is expected not to be a problem in natural mating when spermatozoa are in contact with seminal plasma for a short period of time only. This factor is thought to negatively influence the viability of sperm cells when either ejaculated semen is diluted or seminal plasma is added to epididymal sperm (Shannon 1965).

A reduction of freezability of spermatozoa has been described after mixing with accessory gland secretions in stallions (Magistrini *et al.* 1988). Whereas the pre-incubation of porcine epididymal sperm in seminal plasma has been found to protect the acrosomal membranes from damage during cold shock (Berger and Clegg 1985).

1.4.5 Comparison of ejaculated semen and epididymal sperm

1.4.5.1 Morphology

Spermatozoa undergo changes during their passage through the epididymis. Ejaculated sperm cells differ in their membrane structure as they bind proteins, such as HBP, which are secreted by accessory glands, on their surface. Many spermatozoa stored in the cauda epididymis still have a distally located cytoplasmic droplet which was found to be removed during their further passage through the male reproductive tract. The agent responsible for this was, as stated above, found to be a haemolytic factor called phospholipid-binding-protein (PBP) (Matousek and Kysilka 1980).

1.4.5.2 Fertilizing capacity

Ejaculated bovine sperm cells are in an uncapacitated state which is extremely stable. Release from this state is mediated by heparin and is necessary in order for them to undergo the acrosome reaction (Florman and First 1988). In contrast, bovine epididymal spermatozoa are able to escape from the noncapacitated state in simple salt solution without special capacitating agents and are able to fertilize eggs immediately (First and Parrish 1987, quoting an unpublished paper by J.J. Parrish).

It has been found that there is not necessarily a correlation between the frequency of acrosome reactions and fertility rates when epididymal sperm was used (Ball *et al.* 1983). In this study bovine epididymal sperm was treated with different ways to induce acrosome reaction. It was then found that a high frequency of acrosome reactions was not necessary for fertilization. It has also been established that acrosome reacted sperm cells have a short lifespan (Christensen *et al.* 1996).

A comparison of the in vitro fertilizability of frozen-thawed epididymal spermatozoa with those of ejaculated spermatozoa deprived of seminal plasma and normal ejaculated spermatozoa shows that the efficiency of IVF was lower for the latter two (Katska *et al.* 1996). These results have been confirmed in another study also performed on cattle. But the advantage of a higher fertility rate when epididymal sperm were used also resulted in a higher incidence of fertilization anomalies. Although not proven it was suggested that a higher dose of heparin would prevent this (Pavlok *et al.* 1988).

Measured by the rates of pronucleus formation and cleavage, a better fertilizing capacity in IVF for epididymal boar spermatozoa is described by Rath and Niemann (1997). In experiments described by Haller *et al.* (1980) it has been shown that seminal plasma inhibits the in vitro survival of sperm, at least in the goat.

1.4.5.3 Resistance to cold shock

Berger and Clegg (1985) have found that the acrosomal membranes of epididymal porcine spermatozoa were more resistant than those of ejaculated sperms, measured by hyaluronidase release. These findings concur with those of Johnson *et al.* (1980), who describe in equines a higher membrane permeability of eosin for

ejaculated spermatozoa than for epididymal ones. An explanation of the greater susceptibility of ejaculated sperms to cold shock is given by Wales and White (1959) and may be due to dehydration of the lipid capsule during maturation. They state as well that the resistance to cold shock is mainly a property of the cell and is little affected by the accessory secretions.

This stands in contradiction to the former existing opinion, established by Bialy and Smith (1959) that spermatozoa acquire their susceptibility to cold shock by reception of substances which are present in the secretion of the ampullae.

1.4.5.4 Post-thaw motility

The general accepted opinion is that spermatozoa gain their ability to move in the epididymis. In contrary to this Hopkins (1991) describes a lower motility of epididymal sperm due to the fact that ejaculation has not taken place. Haller *et al.* (1980) as well as Rath and Niemann (1997) describe the post-thaw motility of epididymal sperm to be higher than ejaculated semen in goats and boars respectively. Magistrini *et al.* (1988) made similar findings in the stallion and describes a higher velocity and vitality of frozen thawed epididymal spermatozoa as compared to ejaculated spermatozoa.

1.4.5.5 Availability and problems arising in the collection of either ejaculated or epididymal sperm in African buffaloes (*Syncerus caffer*)

The availability of epididymal sperm from African buffaloes for this study was limited to those bulls culled and hunted as part of an annual population management and disease testing program at Umfolozi-Hluhluwe National Park. . Since these culling and hunting programs are executed during the winter months in South Africa (i.e., May through August), it was not possible to examine if there are any seasonal affects on epididymal sperm morphology, activity and resistance to cryopreservationOne is therefore restricted to the amount of animals that are going to be shot within the hunting season. As this is only during the colder and dryer months of the year a comparison of the influence of season on epididymal sperm cells is impossible.

The possibility of collecting epididymal sperm after castration or death can assure that the genetic material from male individuals can still be preserved after the event of severe acute trauma or even death (Hopkins 1991; Howard *et al.* 1986). This is

substantiated by the findings of Krzywinski (1980) which describe that sperm collected even two hours after the animal's death was suitable for freezing in moose (*Alces alces*) and red deer (*Cervus elaphus*). Gerber *et al.* (2001) collected epididymides 45 min after the death of African buffalos and stored them at 34°C for five hours and at 4°C for another 24 hours. Despite the delay between collecting the epididymides and flushing the sperm acceptable post-thaw motility has been achieved.

On the other hand "do animal species vary in their response to electroejaculation as do individual members of each species and it is as much an art as a science to obtain a successful ejaculation in terms of sperm number and absence of urine" (quoting Seager and Platz 1976). Hopkins *et al.* (1988) suggest that it cannot be taken for granted that an even closely related wildlife species such as the gaur (*Bos gaurus*) would respond to the electroejaculation protocol developed for *B.taurus* bulls in a similar fashion.

African buffalo semen has already been collected by electroejaculation and the used protocol is described by Brown *et al.* (1991). Results according to amount of semen, sperm concentration and percentage of motile spermatozoa are not given in this study.

Bertschinger (1996) also describes semen collection using electroejaculation. When doing so, the drugs used for chemical immobilisation are of great importance and one must be aware that α_1-antagonists inhibit peristalsis of the vas deferens, whereas α_2-agonists have an opposite effect and are therefore recommended. Nevertheless he states various problems arising using this method such as regurgitation of ruminal fluid and consequent pneumonia, as well as overheating.

1.4.6 Influence of various factors to semen and semen parameters

Factors that might influence various semen parameters are age, nutritional status, season and ejaculation frequency. Brown *et al.* (1991) describe that the total number of spermatozoa per ejaculate in African buffaloes is similar between breeding and non-breeding season. What is influenced however is the volume of the ejaculate, which is greater during the breeding season, probably due to an increase in the

secretory activity of the accessory sex glands. In addition Grimsdell (1973) has found no evidence of seasonal changes in male reproduction activity in the African buffalo population in Uganda and suggests therefore that the seasonality of conception may be due to the varying sexual activity in the female only.

Harayama *et al.* (1992) found the percentage of progressively motile spermatozoa as well as the percentage of morphological abnormal spermatozoa collected from the cauda epididymis of Maishan boars, to be at a constant level throughout different times of the year. Therefore it has been concluded that, in the boar, season does not influence the epididymal function in relation to sperm maturation and storage.

König (1998) compared the transmigration rate of the two epididymides of the same animals and found that there was no significant difference for various dilution and media used. He therefore concludes that differences in motilities that arise after treating harvested sperm from each epididymis in different ways are based on these different treatments only.

1.5 Semen evaluation

1.5.1 Motility

The evaluation of sperm motility is an important tool to estimate the fertility of a semen sample and there are several techniques described to measure it. The simplest of these is the estimation of progressively motile cells using a high power microscope (400x) with a stage preheated to about 37°C, referred to as eyeball assessment. The semen should be diluted with as much physiological solution as will ensure that individual cells are visible. This technique is not only simple but also rapid, inexpensive and reliable for an experienced observer.

A more advanced and expensive technique to measure not only the motility but also study the pattern of movement is the so called videomicrography. Spermatozoa are tracked and net displacement velocity, curvilinear velocity of the sperm head, average velocity, the progressiveness ratio, the curvilinear progressiveness ratio and a linear index are measured (Tessler and Olds-Clarke 1985).

In an experiment described by Stewart and O'Hagan (1972) post-thaw motility has been the only factor that showed a significant correlation to fertility. There are probably as many different suggestions for minimum requirements according the post-thaw motility of semen as there are authors. The reason for that is simple as one cannot give a clear dividing line between a fertile or an infertile semen sample according to the motility. Hopkins (1991) sets the minimum standards for wild cattle at a motility of more than 30% immediately after thawing and a decline of no more than half the initial motility after two hours of incubation at 37°C, whereas Katska *et al.* (1996) recommend only to select sperm samples which show above 40% of motile sperm after thawing for IVF trials in bulls.

The requirements for frozen bull semen according to the South African Live Stock Improvement Act state a minimum of 30% linear motile spermatozoa.

The American College of Theriogenologists recommends 30% as a minimum threshold for individual motility before freezing (Chenoweth *et al.* 1993).

Sperm cells are distributed within the female genital tract not only by their own motility, but also by a marked amount mechanically by contractile activity of the female musculature. Sperm motility however is important for the passage through the cervix in species where semen is deposited into the vagina, such as ruminants and primates. Motility is however also important in all species for passing the Utero-tubal junction and also only motile sperm cells are able to penetrate the zona pellucida and therefore fertilize the ovum.

Although sperm motility is necessary for fertilization it is not said that motile spermatozoa are fertile and Hafez (1987) suggests that normal sperm cells lose their fertilizing ability before they lose motility.

1.5.2 Concentration

The concentration of spermatozoa is measured as the amount of sperm cells per ml, or µl in German references. There are obviously marked species differences according to the amount of seminal plasma, which varies within the different domestic animals. The average concentration for bulls is according to Weitze 1,2 million spermatozoa per µl (Weitze and Müller 1991).

There are however no such figures available regarding the sperm concentration of epididymal sperm.

The concentration is usually measured with a standard blood cell haemocytometer. Semen is mixed with a known volume of a spermicidal solution and one drop is added to the haemocytometer. The spermatozoa are counted in the centre and the 4 corner squares (E1-E5) of the major square, termed E. Only those sperm cells within the squares and those that cross the lines at the top and the right hand sides are counted. The number of sperm cells is multiplied by 50.000 as well as the sperm dilution factor (which is usually 20) to obtain the sperm concentration per ml.

1.5.3 Morphology

1.5.3.1 Staining techniques

Morphology is often evaluated in unstained, formalin fixed semen smears using phase contrast microscopy. The routine procedure for morphology is however to evaluate 200 sperm cells in an eosin/nigrosin smear. For more specific details a method to selectively stain the acrosomal membrane has been described in stallions (Cheng *et al.* 1996; Rathi *et al.* 2001; Blottner *et al.* 1998), boars (Flesch *et al.* 1999; Chun-Xia Zou and Zeng-Ming Yang 2000) bulls (Garner *et al.* 1999; Blottner *et al.* 1998; Thomas *et al.* 1997) dogs (Sirivaidyapong *et al.* 2001; Sirivaidyapong *et al.* 2000) and humans (Mortimer *et al.* 1987). It has been proven already that peanut agglutinin (PNA) binds exclusively to the outer acrosomal membrane in boars (Fazeli *et al.* 1997). It has also been reported to bind to the outer acrosomal membrane in cattle, horses, cats and bears. In the later study the results were the same no matter if ejaculated or epididymal and fresh or frozen thawed sperm was used. (Wegner *et al.* 1997). In experiments done on bovine sperm it has been proven that PNA does not bind to live spermatozoa, whereas it specifically binds to the acrosomal region of sperm with a permeabilized plasma membrane (Cross and Watson 1994). In this case the originally intact plasma membrane can be passed by the PNA, hence: the normal sperm cell shows full fluorescence.

Fluorescein isothiocyanate (FITC) is always used as a marker and a fluorescence microscope is used to observe the acrosomal status. The four different stages of the

acrosome reaction as well as the acrosomal integrity that can be differentiated in permeabilized sperm are: 1) a bright fluorescence of the acrosomal cap on an acrosome intact spermatozoon, 2) a disrupted fluorescence of the acrosomal cap of a spermatozoon in the process of breaking down the acrosomal membrane, 3) a fluorescent band at the equatorial region, which indicates leftovers of the acrosomal membrane and 4) no fluorescence at all of a spermatozoon that lost its acrosome.

When the plasma membrane is not permeabilized the pattern will be different. A viable intact sperm cell has an intact plasma membrane. The PNA stain cannot penetrate the plasma membran, hence an intact cell does not stain. Once acrosome reaction starts there is fusion of the plasmamembrane with the outer acrosomal membrane. Then, the outer acrosomal membrane is exposed, hence it is stained (personal communication Colenbrander,B.. Department of Equine Sciences, Section of Reproduction, Yalelaan 12, NL-3854 CM Utrecht, The Netherlands).

A similar technique was used by Kühne (1996), when the influence of seminal plasma on the post-thaw qualities of stallion semen was investigated. In this study Carboxyfluoresceindiacetat (CFDA) was used and acrosomes were only classified as either intact or damaged.

1.5.3.2 Nuclear defects

Defects and malformation of the nucleus automatically lead to an aberration in the head shape, because the nucleus dictates the contours of the sperm head. Two of these defects, namely the pyriform and the tapered heads are, even in bulls with good fertility, not uncommon in small numbers. In both cases a large variation in size of sperm heads can be found. Sperm cells with abnormal head shape are usually found to have good motility and posses normal intact acrosomes. Their fertilising ability however is low.

Not only sperm heads with abnormal shapes but also ones with abnormal size can be found in a sperm population. Microcephalic and macrocephalic heads, which are smaller or larger respectively than normal sperm heads can be found in almost every ejaculate. The incidence of microcephalic heads in bulls with good fertility, however, is less than 1% and those of macrocephalic heads is even lower (Barth and Oko 1989).

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So-called nuclear vacuoles have been described in bull spermatozoa by Slizynska and Slizynski (1953). Bane and Nicander (1966) have found that this defect is actually an invagination of the nuclear membrane into the nucleoplasm and they suggest a tendency towards uncontrolled growth in the acrosomal system to be the reason for this defect. The incidence of such a malformation is less than 1% for bulls with good fertility, but can reach up to 100%, leading to very poor fertility or even infertility(Miller *et al.* 1982).

1.5.3.3 Acrosomal defects

One of the acrosomal defects which is not uncommon in *Bos taurus* bulls is the so called "knobbed acrosome defect". It consists of a excess of acrosomal matrix, which leads to a folding of the enlarged acrosome over the sperm head. The defect is particularly often found when a bull is affected by a disturbance in spermatogenesis. Such affected spermatozoa are considered infertile and animals with a high percentage of knobbed spermatozoa are virtually sterile (Barth and Oko 1989; Blom and Birch-Andersen 1962).

Acrosomal defects cannot only be determined by examination of the spermatozoa under light microscopy but also by alterations of the acrosomal membrane, monitored by the release of hyaluronidase (Triana *et al.* 1980). Guidelines for quality standards in the gaur (*Bos gaurus*) and wild cattle are given by Hopkins *et al.* (1988) and Hopkins (1991). According to these papers, there must not be more than 10% primary and 30% total abnormal cells. Furthermore are more than 50% intact acrosomes after 2 hrs of incubation required.

1.5.3.4 Midpiece defects

The most common defect occurring in this region of the sperm cell is the distal midpiece reflex, which is found in small percentages in the ejaculate of almost every bull, with a varying incidence from 1% to 90%. The spermatozoon typically appears in the shape of the letter J, because of a bend in the distal region of the midpiece. However, there are different variations of this defect found and the principal piece may coil around the bent midpiece or there may be a second bend in the opposite direction above the first bend. Barth and Oko (1989)describe this defect to be temporary and due to adverse environmental influences. Bishop *et al.* (1954) suggest

that this defect might rather be an artefact caused by a failure to protect the sperm from damaging effects of rapid cooling. Although it is unlikely that spermatozoa affected in such a way would penetrate the zona pellucida and would therefore cause a reduction in fertility, no research has been done to prove this (Barth and Oko 1989).

A defect which may be seen quite commonly in ejaculates of bulls, but seldom exceeds an amount of more than 5%, was first described by Blom (1966) as folding and coiling of the tail and named after the bull where it was found as the "Dag defect". It is associated with a fracturing of the axonemal elements in the midpiece as well as a disrupted arrangement of the mitochondria. Ackerman *et al.* (1994) describe in a study a comparatively high average percentage of 8,3% dag defects in 10 African buffaloes which showed nevertheless 87,4% normal spermatozoa.

Small gaps along the mitochondrial sheath, which result in midpiece segments of various lengths, can exceptionally be found in bulls. No cases of infertility were assigned to this so-called segmental aplasia of the mitochondrial sheath (Barth and Oko 1989).

The corkscrew defect, described by Blom (1959) is characterized by an irregular distribution of mitochondria along the length of the mitochondrial sheath. Holt (1982) suggests that it is linked to the retention of the proximal droplet. This defect is only found in dead cells and is often accompanied by other major defects.

1.5.3.5 Tail defect

The most common forms of tail defects are abaxial and accessory tails, which are believed to be closely related forms and often, but not constantly, occur together. Although there is no proof yet, Barth suggests that these defects are inherited (Barth and Oko 1989). Bulls producing these defects usually have semen with both good motility and normal density as well as good post-thaw viability. A difference between the two defects may however be seen regarding their aptitude to fertilize ova and induce embryonic development, because this does not seem to be impaired for spermatozoa with abaxial tails. Sperm cells with accessory tails on the other hand are infertile.

Tail stump defects as described by Arriola *et al.* (1985) have a very low incidence, and the percentage of motile cells within an affected ejaculate is obviously very low. As such bulls usually produce a high percentage of defective cells as well, they are sterile.

As mentioned earlier a comparatively high amount of cytoplasmic droplets can be found in epididymal spermatozoa, but these small spherical masses of cytoplasm are usually only present in a very small amount in ejaculated spermatozoa. Such cytoplasmic relicts surrounding the midpiece just proximal of the annulus are called distal droplets and are not considered as a serious problem. Proximal droplets, which are situated around the neck and proximal midpiece region, may however be a sign of abnormal spermiogenesis and are often associated with many other sperm defects (Barth and Oko 1989).

1.5.4 Longevity

In earlier experiments cryopreservation methods were mostly evaluated according to the post-thaw qualities of frozen-thawed semen immediately after thawing. In more recent studies various authors showed that an evaluation of semen is more meaningful when semen is stored at a certain temperature for several hours and sperm parameters are observed regularly.

Correa and Zavos (1995) for example compared two different dilutions for frozen-thawed semen. The percentage of motile spermatozoa was equal, straight after thawing, but significant differences in motility were seen after 2 hours of incubation at 37°C. This indicates that methods, which seem to result in the same post-thaw motility, can show marked differences when evaluated some time after thawing again.

Senger *et al.* (1976) have also found only a few differences in motility when comparing two different thawing rates immediately after thawing, but significant differences after 4 hours of storage at either 20°C or 37°C. They also state that due to the very low motility after 8 hours of post-thaw incubation, little emphasis can be placed on differences found at that time.

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1.5.5 Separation

To yield a high number of motile spermatozoa and to further enhance their motility various methods have been established. These methods not only separate motile sperm cells from immotile but also stimulate spermatozoa to move.

The most common method is the so-called swim-up procedure, where spermatozoa are covered with TALP or similar media. This can be done either before or after centrifugation.

When using the Percoll technique a high percentage Percoll solution (the content varies from 80% to 90% to 95% according to Morales *et al.* (1991), Menkveld *et al.* (1990) and McClure *et al.* (1989), respectively) forms the bottom layer. A Percoll solution with a lower content (40% Morales *et al.* (1991); Menkveld *et al.* (1990) or 47,5% McClure *et al.* (1989)) covers the first layer. The semen sample is put on top of that and the whole tube is centrifuged. After that the semen pellet will be collected from the bottom of the tube and washed by further centrifugation with a fresh diluent.

Comparison of Percoll and swim-up separaton were made by Morales *et al.* (1991) and show that the Percoll technique resulted in a higher number of total as well as motile spermatozoa. Both methods produce functionally normal spermatozoa as both sperm populations were equally capable of binding to the hamster zona pellucida and undergoing the acrosome reaction. According to the functional integrity of sperm cells, evaluated by the hypo-osmotic swelling test (HOS), no differences have been found after preparation with the Percoll and with the swim-up (Cheek *et al.* 1992).

Less common methods include the swim-down separation and the glass-bead column seperation (Kobayashi *et al.* 1991 and Lechtzin *et al.* 1991 respectively). The latter was compared with the swim-up method by Calamera *et al.* (1991) and a higher percentage of motile spermatozoa has been found when using the glass beard column. However, the velocity and linearity of these forms was less than when using the swim-up technique.

A more recent study describes a new method for separating bovine frozen-thawed semen (Shamsuddin and Rodriguez-Martinez 1994). The conventional swim-up method was modified in such a way that an extra layer of hyaluronic acid (HA)

medium was put between the semen and the TALP medium. When compared with the swim-up technique, the HA-selected spermatozoa showed a better motility and uniformly cleaned membranes and the percentage of sperm cells with an intact membrane and acrosome increased. These spermatozoa can therefore be used for IVF immediately, without any further washing by centrifugation or rinsing.

1.6 Collection of epididymal sperm

1.6.1 Influence of time

When sperm is collected from the epididymis of dead animals attention must be paid to the time between the death of the animal and the collection of the sperm or epididymis. Another factor which has to be taken into consideration, especially when dealing with wildlife under field conditions, is the time between the collection of the sperm or epididymis and the further processing steps done at a laboratory.

Hopkins *et al.* (1988) recommend that the spermatozoa must be recovered immediately post-mortem due to the deleterious effects of tissue degeneration, but these effects can be bypassed by transporting the collected epididymis cooled on ice (4°C) as described by Hopkins (1991) and Lambrechts *et al.* (1999). Concurrent to these reports, experiments of Gerber *et al.* (2001) prove that there are no differences between 5 and 29 hours (another 24h at 4°C) of storage, according to the motility. When acrosomal integrity was compared, there was, although not significant, a trend towards more acrosome defects after 29 hours.

1.6.2 Influence of temperature

The epididymis can be flushed immediately after death of the animal and therefore at a temperature of approximately 35°C. A collected epididymis can also be stored on ice and transported to a laboratory. The collection of the spermatozoa would then take place at about 4°C. Jun Tao *et al.* (1995) describe this procedure and found a higher post-thaw motility of spermatozoa of mice, when they are collected at cold temperatures.

1.6.3 Influence of collection method

As mentioned earlier there are two different methods of harvesting the spermatozoa, namely mincing and flushing. When epididymal sperm is collected by mincing, the epididymis is placed on a petri dish and cuts are made into it using a scalpel so that the spermatozoa and epididymal fluid can ooze out. Extender diluent is added afterwards. When epididymal sperm is flushed extender is injected retrograde into the ductus deferens with needle and syringe. As the lumen distends stab incisions are made into the cauda epididymis and spermatozoa and extender are collected (personal communication Gerber, D. Section of Reproduction, Faculty of Veterinary Science, University of Pretoria, South Africa). Both techniques are described in the literature (Braun *et al.* 1994; Graham 1994; Hopkins *et al.* 1988; Jimenez 1987; Katska *et al.* 1996; Lambrechts *et al.* 1999; Lengwinat and Blottner 1994; Loskutoff *et al.* 1996; Nakagata and Takeshima 1992; Rath and Niemann 1997) but no research has been done yet to compare them.

1.7 Dilution of epididymal sperm

1.7.1 Media

1.7.1.1 Media used previously in African buffaloes

There are probably almost as many different media used as there are attempts made to cryopreserve semen from various species. From what has been done in African buffaloes and closely related species it has been shown that Triladyl™ (Minitüb, Germany) yields better post-thaw acrosome-integrity results than sperm-Tyrodes's-albumin-lactat-pyrovate (sperm-TALP) media with or without glycerol (Lambrechts *et al.* 1999).

Sperm-TALP (Bartels *et al.* 1996), and Triladyl™ (Minitüb, Germany) (Bartels *et al.* 1996, Gerber *et al.* 2001 and Gerber *et al.* 2002) have also been used by other authors. The total post-thaw motilities achieved in these studies have been between 19% and 45%.

1.7.1.2 Egg yolk free diluents

Semen extenders containing animal products always bear the risk of being contaminated with bacteria or mycoplasma. These can be a source of endotoxins, which are capable of negatively influencing semen quality. The Office International des Epizooties recommends the use of egg yolk from SPF (specific pathogen free) flocks or milk heat-treated at 92°C for 3-5 minutes for the dilution of boar semen (OIE Terrestrial Animal Health Code 2003, APPENDIX 3.2.3., Article 3.2.3.5). When using egg yolk in semen extenders for bovines the seperation from the eggs should be done using aseptic techniques. As an alternative the use of commercial egg yolk prepared for human consumption or egg yolk treated by pasteurisation or irradiation is recommended (OIE Terrestrial Animal Health Code 2003, APPENDIX 3.2.1., Article 3.2.1.9). Antibiotics added to semen extenders do not prevent the growth of all bacteria. Thus bacteria were found in the final preparations of egg yolk-based and milk-based semen extenders. No contamination was found in an animal product free semen extender (Bousseau *et al.* 1998). In addition, egg yolk, as well as milk, contain steroid hormones and their precursors (Hartmann *et al.* 1998, Möstl *et al.* 2001) which may affect the fertilizing capacity of spermatozoa. A study conducted on human spermatozoa shows that progesterone causes a hyperactivation of sperm cells and hence a decrease in linear motility and a following accumulation (Jaiswal *et al.* 1999). Because the composition of egg yolk varies slightly, semen extenders containing egg yolk can never be totally standardized (Müller-Schlösser *et al.* 2001). Therefore, animal product-free, totally defined semen extenders for cattle have been developed (e.g. AndroMed®, Minitüb, Germany; Biociphos® plus, IMV, L`Aigle, France).

Semen parameters and in vivo as well as in vitro fertility obtained with AndroMed® ™ (Müller-Schlösser *et al.* 2001) or with the lecithin-based extender Biociphos® plus (Müller-Schlösser *et al.* 1995; Hinsch *et al.* 1997; Bousseau *et al.* 1998) were comparable to those obtained with milk powder or egg yolk containing extenders in cattle

When in-vitro and in-vivo fertilizing abilities of semen frozen with the three above mentioned extenders were compared. No differences could be found then (Bousseau *et al.* 1998).

In another study Biociphos® (I.M.V. L'Aigle, France) yielded results at least as good as a conventional TRIS based extender in respect of total and linear motility, velocity and acrosomal damage (Müller-Schlösser *et al.* 1995).

It has also been shown in bulls that another commercial egg yolk-free semen extender AndroMed ®, Minitüb, Germany) can produce equal sperm parameters and fertility rates (Müller-Schlösser *et al.* 2001).

When a soybean based synthetic extender was compared to Triladyl™, a significantly higher cleavage rate and percentage of hatched blastocysts have been found for the egg yolk free diluent (Palasz *et al.* 2002).

1.7.1.3 Additives

Effects of additives such as sodium triethanolamin lauryl sulphate (Equex STM) or sodium and ethanolamine lauryl sulphate (Orvus ES) have been studied in different species by various authors (Arriola and Foote 1987; Bhosrekar *et al.* 1990; Graham *et al.* 1971; Jimenez 1987; Pursel *et al.* 1978; Rota *et al.* 1997). In semen from bulls and water buffaloes a beneficial effect according to the percentage of forward motile spermatozoa has been found (Bhosrekar *et al.* 1990). These findings concur with those of Arriola and Foote (1987), who proved a concentration of 0.25 to 0.5 % Equex STM to increase the percentage of motile sperm cells in bulls. They also show that concentrations of 0.5% and 1.0% were optimal for sperm survival and that an increase of the concentration up to 2% had no negative effects. Further experiments on the effect of Equex STM on bull semen performed by Foote and Arriola (1987) show that the additive increases the number of motile sperm cells using any one of three different thawing rates. Pursel *et al.* (1978) describe beneficial effects of Orvus ES on boar semen. A higher number of sperm cells with acrosomes with a normal apical ridge as well as a higher percentage of motile spermatozoa has been found. It is concluded in this study that 1 to 1.5% Orvus ES is the optimum for the acrosomes and a concentration of 0.5 to 1% results in the highest post-thaw motility. It has been found in the same study that Orvus ES increases the resistance of sperm cells to faster cooling protocols . Concluding from their experiments, they support the hypothesis of Graham *et al.* (1971) that Orvus ES acts through alteration of egg yolk constituents. Graham *et al.* (1971) have also previously stated that the protection

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might be either due to an action on the egg yolk, the seminal plasma, deleterious components or on the sperm wall. A beneficial effect of Equex STM has also been found on frozen-thawed dog semen, as it enhances viability as well as longevity (Rota *et al.* 1997).

Experiments performed by Jimenez (1987) show that Equex STM has no such effects on the motility of epididymal stallion spermatozoa.

1.7.1.4 Cryoprotectants

In relation to cryoprotectants, Loskutoff found that out of glycerol, ethylene, glycol, propylene glycol and dimethyl sulfoxide the least toxic for various African antelope species is glycerol and that a concentration of 8% v/v is more protective than one of 4% v/v (Loskutoff *et al.* 1996). Nevertheless one should aim for a low glycerol concentration, which minimizes the toxic effects but still exerts an antifreeze action (Penfold and Moore 1993).

1.7.2 One-step or stepwise dilution

There are different options regarding the time of addition of the cryoprotectant. This can be done either before or after cooling the spermatozoa. The so called stepwise method, where the cryoprotectant is added after cooling only, has been shown to be superior to the one step-method, when comparing the sperm motility of bovine semen (Arriola and Foote 1987). Other experiments performed on bulls and described by Foote (1970) show that there is no difference in either sperm motility or fertility whether glycerol is included in the initial diluent or only added after cooling to 5°C. When glycerol was added either to the freshly collected semen or after cooling to 20°C, 10°C or 5°C no differences in non return rates could be found in cattle (Graham *et al.* 1958).

Braun *et al.* (1994) as well found no effect on post-thaw motility of epididymal spermatozoa when comparing the two different methods in stallions. Wilmut *et al.* (1973) suggest that it cannot be stated whether the one-step or two-step method is superior for pelleted boar semen. They proved, that there is a significant correlation between the dilution method, required glycerol concentration and cooling rate.

Whereas an increased concentration of glycerol has a detrimental effect when using the one-step dilution, it proved to be beneficial in the two-step method.

1.7.3 Spermconcentration

The suitable number of live and motile sperm cells per insemination unit may vary from species to species and recommendations for the African buffalo are not available yet. For wild cattle a recommended number of 60 to 80 million spermatozoa per ml has been given by Hopkins (1991), with Drost (1991) advising the dilution of water buffalo semen to such an extent that there are 12 to 15 million actively motile spermatozoa per insemination unit after thawing.

According to the South African Live Stock Improvement Act, at least 7 million live sperm cells per straw, of either 0.25 or 0.5 ml are required.

1.8 Equilibration and cooling

1.8.1 Equilibration time

The time spermatozoa are exposed to the cryoprotectant prior to freezing has different effects on its post-thaw characteristics in different species. Jimenez (1987) for example found no difference in the post-thaw motility of samples of eight equids exposed to different pre-freeze equilibration times. Whereas Dhami and Sahni (1993) describe a significantly increased post-thaw motility as well as fertility for an equilibration period of 2 hours at 5°C for taurine bull spermatozoa, compared to no equilibration time. Berndtson and Foote (1969) on the other hand found that the shortest exposure time to glycerol gave the highest percentage of sperm survival for bull semen and O´Dell and Hurst (1956) have found that the percentage of motility is the higher the shorter the exposure to glycerol, but stated that remarkable variations exist amongst different bulls.

It has been established that the equilibration time is, at least in bulls, dependant on the cooling rate (Ennen *et al.* 1976). The slower spermatozoa are cooled, the less equilibration time is required to gain optimal motility. The results of the study described by Wiggin and Almquist (1975a) stand in contradiction to the above

mentioned. They found that there is no significant difference in the percentage of intact acrosomes and motile spermatozoa in bovine semen samples when equilibrated for 0.5 or 2 hours. In another study described by Wilmut *et al.* (1973) it has been proven that the faster semen was cooled, the higher glycerol concentrations were needed in boars. Furthermore the longer the spermatozoa were exposed to glycerol the lower the concentration needed to gain the same results in post-thaw semen quality.

A significant correlation between thawing rate and glycerol equilibration time has been found only for intact acrosomes but not for sperm motility. In another study it is stated that glycerol equilibration times that exceed 4 hours are harmful to post-thaw sperm motility (Wiggin and Almquist 1975b).

Crabo *et al.* (1980) describe a higher motility of water buffalo semen after an equilibration time of 7 hours, compared to 5 hours.

1.8.2 Cooling rate

As for many other factors in the cryopreservation procedure the cooling rate influences the post-thaw motility, the vitality and the number of damaged sperm cells. The cooling rate is the decrease in temperature of the semen sample from body temperature down to 4°C in relation to time. Braun *et al.* (1994) describe that five different cooling protocols have no significant effect on the post-thaw motility of epididymal spermatozoa in stallions. The results presented by Dhami and Sahni (1993) show an increased post-thaw motility and fertility, when slow cooling bovine semen for 2 hours from 30°C to 35°C down to 5°C, instead of just one hour. For bovine semen, cooling over a period of 2 hours has been found to be superior to cooling over 0.5 hours (Ennen *et al.* 1976).

1.9 Freezing and thawing

1.9.1 Freezing rate

Like many other steps in cryopreservation, both contradictory opinions and results exist regarding the optimal freezing rate. Grund *et al.* (1980) describe the existence

of a critical temperature range between –13°C and –35°C and state that a fast cooling velocity at this point is harmful to sperm cells. The motility of bull sperm was higher for slow freezing rates than it was for fast ones.

Studies by Mortimer *et al.* (1976) show that there is no difference when bovine semen is frozen from 5°C to –130°C within 2,3,5,7,12 or 20 min, but post-thaw motility is lower when freezing takes place within only 1 min. Experiments described by Jondet *et al.* (1980) show higher sperm survival rates when semen is not plunged immediately into liquid nitrogen but frozen in liquid nitrogen vapour beforehand to a temperature of at least –80°C.

1.9.2 Straw size

Jenichen (1991) describes that insemination doses of 0,25 ml lead to a significant increase of the non-return rate compared to 0,5 ml doses in bulls, which is concurrent to the findings of Berndtson and Foote (1969) that show a higher number of live sperm cells in straws than in ampoules.

1.9.3 Pellets

The cryopreservation of bull semen in pellets was common in some Eastern European countries as well as Finland and Israel. The advantages of this method were that semen was diluted with an easy producible dilution and less liquid nitrogen was used. The disadvantages on the other hand were described to be that the semen was more difficult to put in place, the storage volume was less satisfactory than it is for straws and the identification of the doses stored was more difficult (Delaunois 1980). Findings of Adler *et al.* (1968) that show that there is a higher variation among technicians using pellets than for straws must also be regarded as a disadvantage. They also found a lower breeding efficiency when using pellets, whereas Jenichen (1991) describes the fertilization rates to be equal to those reached by using straws of different sizes. Considering the survival rate Berndtson and Foote (1969) found pellets of bull semen to be superior compared to ampoules, as well as straws. Due to all these disadvantages and the low hygiene standards, pellets are generally no longer used.

1.9.4 Thawing rate

The opinions on whether frozen semen should be thawed rapidly, slowly or at a comparable rate as it was frozen are as contrary as the results of different experiments. Jun Tao *et al.* (1995) compared four different thawing protocols and found that only the highest used temperature has an adverse effect on the motility of mouse spermatozoa. On the other hand Loskutoff *et al.* (1996) found rapid thawing to result in higher post-thaw motility rates in blesbok and impala. Senger *et al.* (1976) also describe a higher percentage of intact chromosomes following thawing at 35°C for 1 min compared to thawing at 5°C for 3 min. Brown *et al.* (1982) proved further that thawing at 35°C for only 12 s leads to an internal straw temperature of –4°C only and that the delay that follows of warming the semen to approximately body temperature is detrimental to the viability of sperm cells. Lambrechts *et al.* (1999) however describe no influence of thawing rate in African buffaloes. A comparison of thawing of bull semen at 4°C, 20°C, 35°C and 75°C in water as well as 20°C in air, made by Aamdal and Andersen (1968) shows that the increase of the survival rate is positively related to an increase in the thawing temperatures. This was confirmed by Arriola and Foote (1987), Jenichen (1991), Pace *et al.* (1981) and Wiggin and Almquist (1975a), who have all found higher thawing temperatures to be superior for bull semen.

Experiments described by Wiggin and Almquist (1975b) show that higher thawing rates are superior to lower, as long as the thawing temperature does not exceed 76°C.

2 MATERIALS AND METHODS

2.1 Model system and justification of the model

It is important to be able to successfully freeze sperm from the African buffalo. It allows the storage of genetics of valuable animals e.g. from animals with exceptionally large horns, and it allows the transport of such genetics to any place in the world where it can be used at any time. An aliquot of frozen sperm as well as smears or blood samples can be taken and examined for the presence of certain diseases to ensure that no diseases are transmitted with the genetic material. It is

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easier to obtain epididymal sperm during culls or hunts than to obtain semen by electro-ejaculation.

2.2 Experimental procedures

2.2.1 Collection and processing of epididymides and spermatozoa

Epididymides were collected from 11 African buffalo bulls culled during a disease eradication program in Huhluwe/Umfolozi National Park in September 2001. This program was performed under the responsibility of the KwaZulu Natal Nature Conservation Service. Only adult bulls with 3 or more pairs of permanent lower incisors (about 3 to 3.5 years of age) were used in the trial.

The testes and epididymides from each bull were collected trough a scrotal incision within 10 minutes after culling. The vas deferens, the cauda epididymidis and part of the corpus epididymidis were dissected free from the testis. The epididymal duct was then dissected free and cut at the site in the tail of the epididymis where the tubular diameter becomes distinctly larger distally. A blunted 23 or 25 G needle connected to a 10 ml syringe was then inserted into the vas deferens and the sperm flushed with air in a retrograde direction.

The harvested sperm of both epididymides of the same bull were mixed and split into two aliquots, each of which was poured into a 15 ml plastic tube, containing 13 ml of the two different cryodiluents. The two semen extenders used were Triladyl™ and AndroMed® (both Minitüb, Germany). The glycerol concentrations of these media are 6.6% and 6.8% respectively. The concentration of the sperm/diluent mixture was determined with a haemocytometer and was between 100 and 150 spermatocytes per ml.

The sperm/diluent mixture was mixed and the two 15 ml tubes were put into a 500 ml water bottle containing water at a temperature of 20°C. The water bottle was placed into a Styrofoam box, containing water and ice cubes in such a relationship that the temperature of the water was 4°C, resulting in a cooling rate of 0.25°C/min. The samples were then transported to a laboratory.

The original difference was attributed to handling mistakes and the semen with poorer motility was discarded.

2.2.5.2 Longevity

The longevity of the sperm was assessed by evaluating the total and progressive motility of the equilibrated sperm. Sperm samples, which were kept in a water bath at 37°C, were examined at 0 (t0), 1 (t1) and 2 (2t) hours after thawing.

2.2.5.3 Acrosomal integrity

The acrosomal integrity was evaluated after a storage time of 5 months. The content of a thawed 0.25 ml straw was emptied in an Eppendorf tube. The sperm diluent mixture was washed twice with 200 µL of PBS by centrifugation at 600 G for 5 min. The supernatant was removed and the pellet re-suspended with 200 µL of PBS. 50 µL of IGEPAL CA-630 (Sigma-Aldrich LTD, Atlasville, South Africa, cat no: D1626) and 50 µL of EthD-1 (Ethidium Homodimer, Laboratory Specialist Services LTD, Clareich, South Africa, cat no: E-1149) were added and incubated for 5 min at 37°C to permeabilize the sperm membrane and to stain the spermatozoa respectively. Then 5 µL of 1mg/ml ssDNA (Deoxyribonucleic acid sodium from Salmon, Sigma-Aldrich LTD, Atlasville, South Africa, cat no: D1626) were added to compete for the unbound EthD-1 and it was further incubated for 1-2 min. Spermatozoa were fixed by addition of 50 µL of 4 % Paraformaldehyde and 1 % Glutaraldehyde in PBS and kept in the dark for 1 min. Then the solution was washed once with 200 µL of PBS by centrifugation at 600 G for 5 min. The supernatant was removed and the pellet re-suspended with 50 µL of PBS. 50 µL (1:1) of FITC-PNA (Lectin from Arachis Hypogaea*Fluorescein, Sigma-Aldrich LTD, Atlasville, South Africa, cat no: L7381) were added and incubated for 10 min at 37°C. It was again washed once with 200 µL of PBS by centrifugation at 600 G for 5 min, the supernatant removed, re-suspended with 25-100 µL of PBS and kept in the dark.

Equal amounts of 2 µL spermsuspension and antifade (Slowfade Antifade Kit, Laboratory Specialist Services LTD, Clareich, South Africa, cat no: S-2828) were put on a microscopic slide and covered with a cover slip. The samples were examined under the epifluorescence microscope (BH2-RFCA; Olympus, Tokyo, Japan) at a magnification of x 400-1000.

2.2.2 Evaluation of fresh sperm

Before further processing the motility was assessed from each solution by means of eyeball assessment using a phase contrast microscope. Percentage of progressive and aberrant motile sperm cells were recorded.

2.2.3 Freezing of sperm

After 2 hours five 0.25 ml French straws were loaded from each of the two sperm dilutions and placed 4 cm above liquid nitrogen for ten minutes before being plunged (ET2). They were then stored in goblets in a liquid nitrogen container until thawing.

The 15 ml tubes with the remaining sperm dilutions were further kept in water bottles in a water bath at 4°C.

The procedure of packing and freezing straws was repeated 8 times after every hour (ET 3-9)

2.2.4 Thawing of sperm

After a storage time of between 4 and 37 days the straws were thawed in water at a temperature of 37°C for at least 30 seconds. The contents of two straws of the same treatment were emptied into two different pre-heated (37°C) 3 ml Perspex-tubes and stored in a water-bath at 37°C.

2.2.5 Evaluation of the post-thaw sperm quality

2.2.5.1 Motility

Twenty five micro litres of the thawed sperm were further diluted with 100 µl of the corresponding diluent. The motility of the sperm from both thawed straws was determined by eyeball assessment using a phase contrast microscope. In three cases the motility differed by 10% or more (10%, 10% and 15% respectively). From these three batches a third straw was thawed and the motility evaluated. The motility was in all cases similar to the motility of the better one of the first two straws thawed.

Spermatozoa were classified according to their appearance as acrosome intact, acrosome damaged or as they had lost their acrosome. One hundred spermatozoa of each sample were evaluated and the results expressed as percentage.

2.2.6 Statistical analysis

2.2.6.1 Equilibration times

A "Two Way Repeated Measures ANOVA" was used to test for differences between the eight different equilibration times separately for the use of AndroMed® or Trilady™. Total and progressive motility were compared separately at any time of their measurement. In the case that normality failed a "Friedman Repeated Measures Analysis of Variance on Ranks"-Test was used.

2.2.6.2 Media and Longevity

To compare the two different media when the equilibration time was the same for both, a paired t-test was used. In the case that normality failed a "Wilcoxon Signed Rank Test" was used. After it became evident that equilibration time does not influence sperm motility the mean values for all equilibration times were used for each buffalo and motilities before freezing, immediately, one and two hours after thawing were compared amongst each other by a "Repeated Measure One Way ANOVA". To identify different groups the Tukey test was used.

3 RESULTS

Total and progressive motilities of fresh sperm samples varied between the bulls and averaged 58 ± 17 % (mean ± SD, range 33-80 %) and 35 ± 21 % (range 10-60 %) respectively.

Different equilibration times did not influence the post-thaw motility, when using Triladyl™. The results for total motility immediately after thawing varied from 49 ± 13 % (mean ± SD) to 59 ± 8 % for an equilibration time of 8 hr and 4 hr respectively. Progressive motility values measured at the same time (t0) were between 10 ± 8 % after 2 hr of equilibrating and 23.3 ± 7.67 after 4 hr (see FiguresFigure 3.1,Figure 3.3Figure 3.5).

Different equilibration times did also not influence the post-thaw motility, when using AndroMed®. The results for total motility immediately after thawing varied from 42 ± 20 % to 51 ± 14 % for an equilibration time of 5 hr and 4 hr respectively. Progressive motility varied from 11 ± 10 % to 19 ± 13 % for 2 and 7 hr of equilibration time respectively (see FiguresFigure 3.2Figure 3.4 andFigure 3.6).

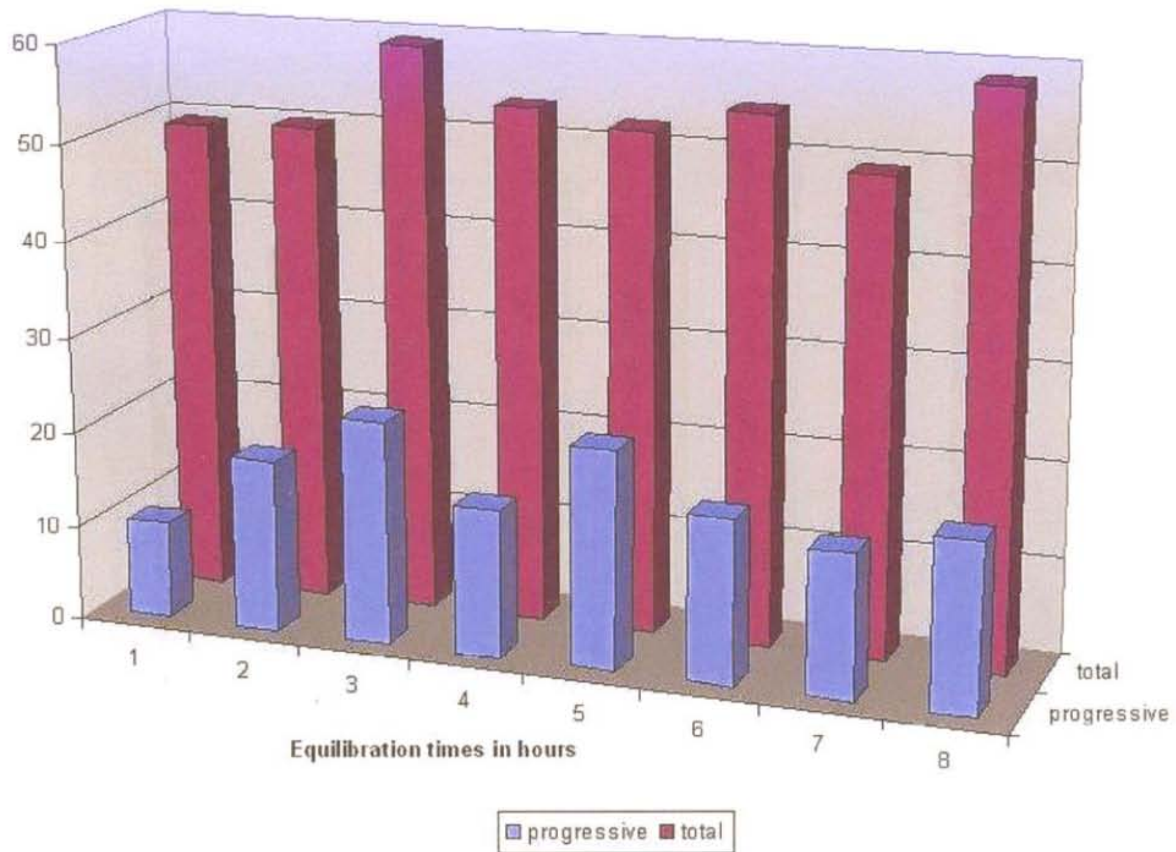

Figure 3.1 Progressive and total motility for epididymal sperm frozen with Triladyl immediately after thawing (mean values of samples taken from 11 buffaloes)

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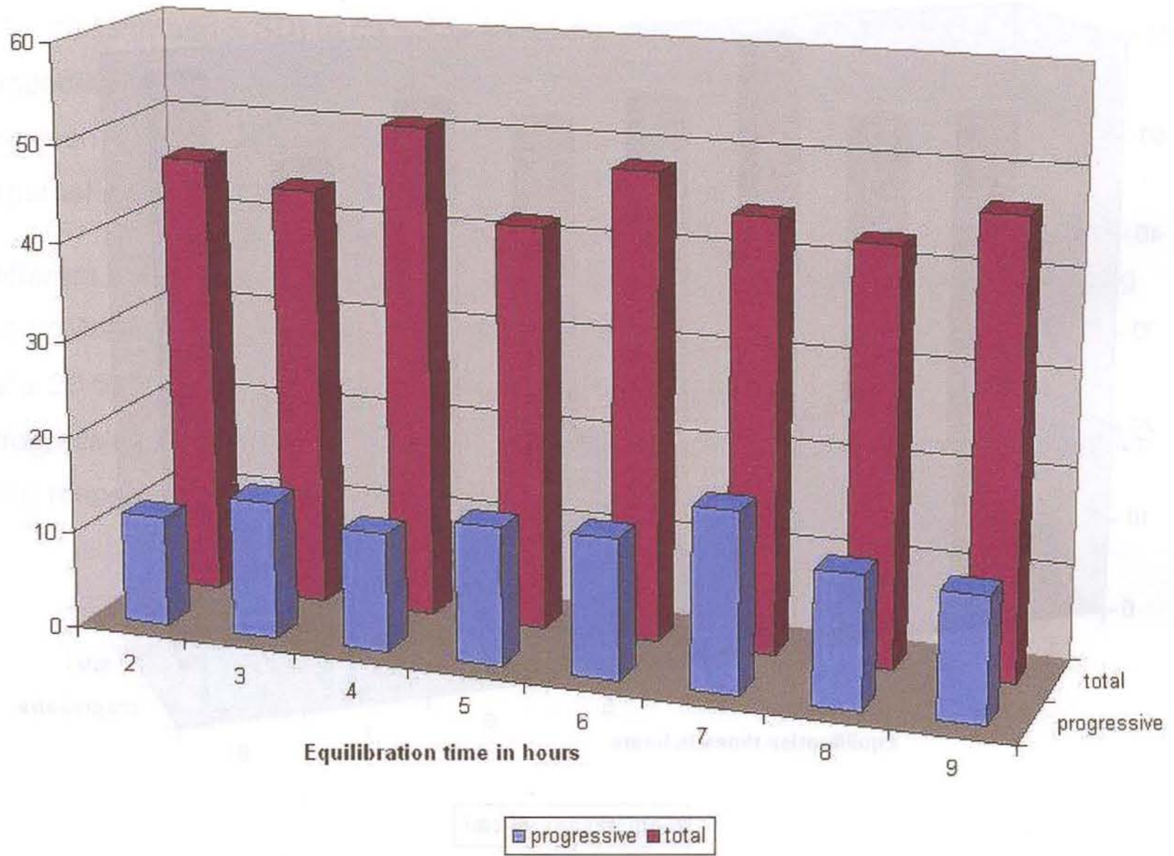

Figure 3.2 Progressive and total motility for epididymal sperm frozen with AndroMed immediately after thawing (mean values of samples taken from 11 buffaloes)

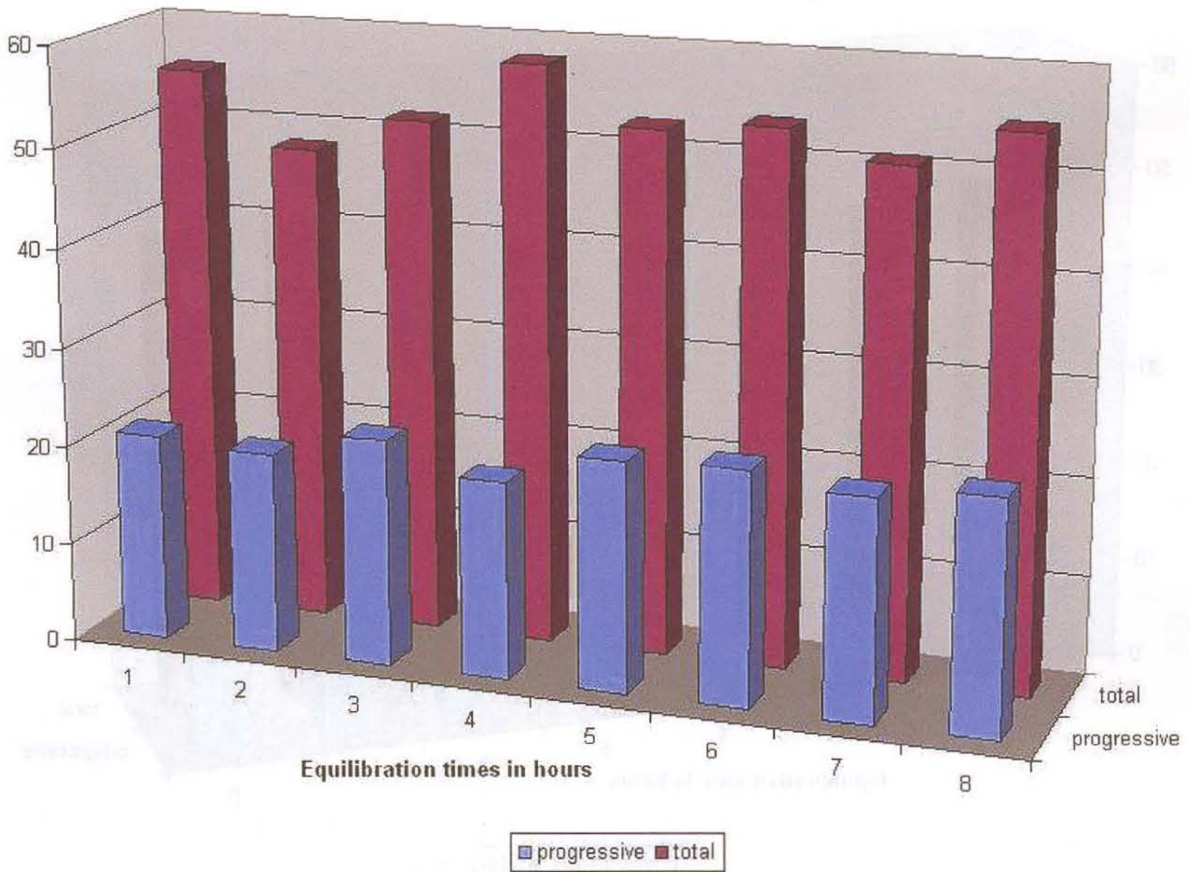

Figure 3.3 Progressive and total motility for epididymal sperm frozen with Triladyl one hour after thawing (mean values of samples taken from 11 buffaloes

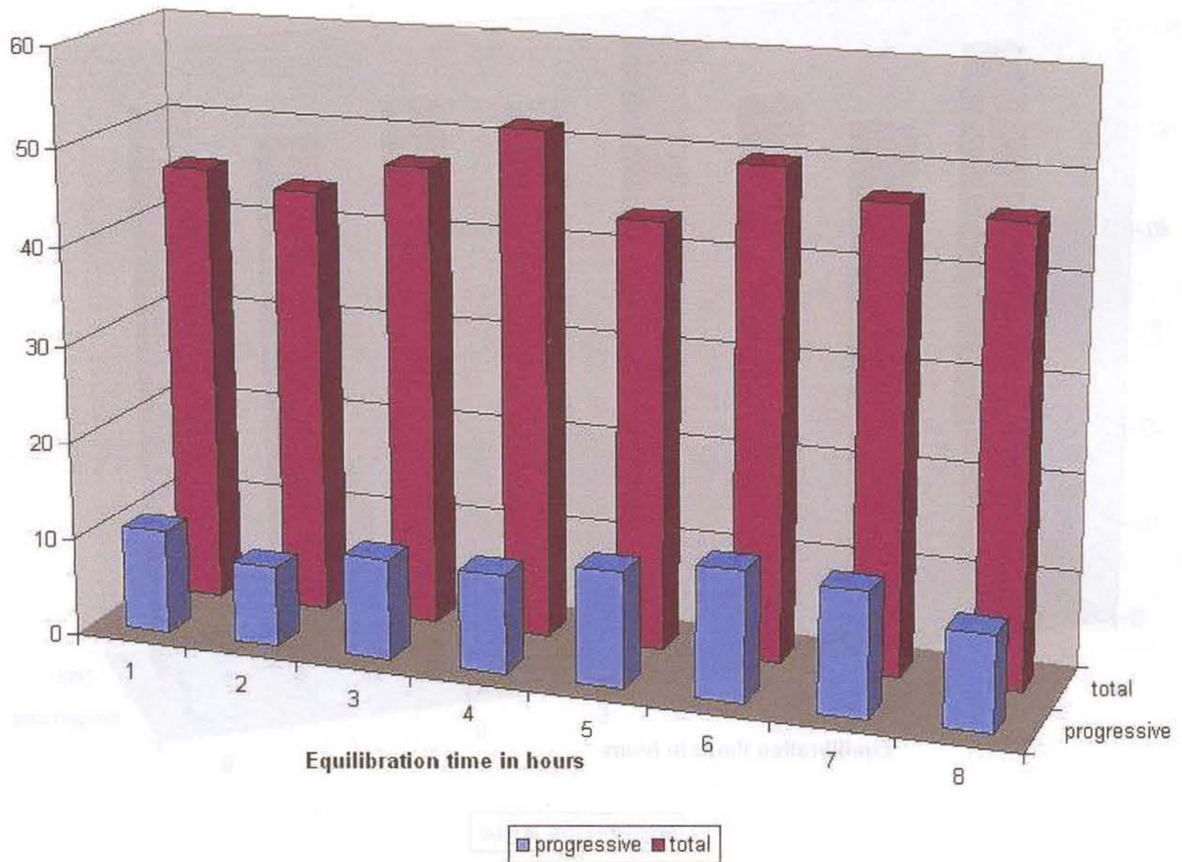

Figure 3.4 Progressive and total motility for epididymal sperm frozen with AndroMed one hour after thawing (mean values of samples taken from 11 buffaloes)

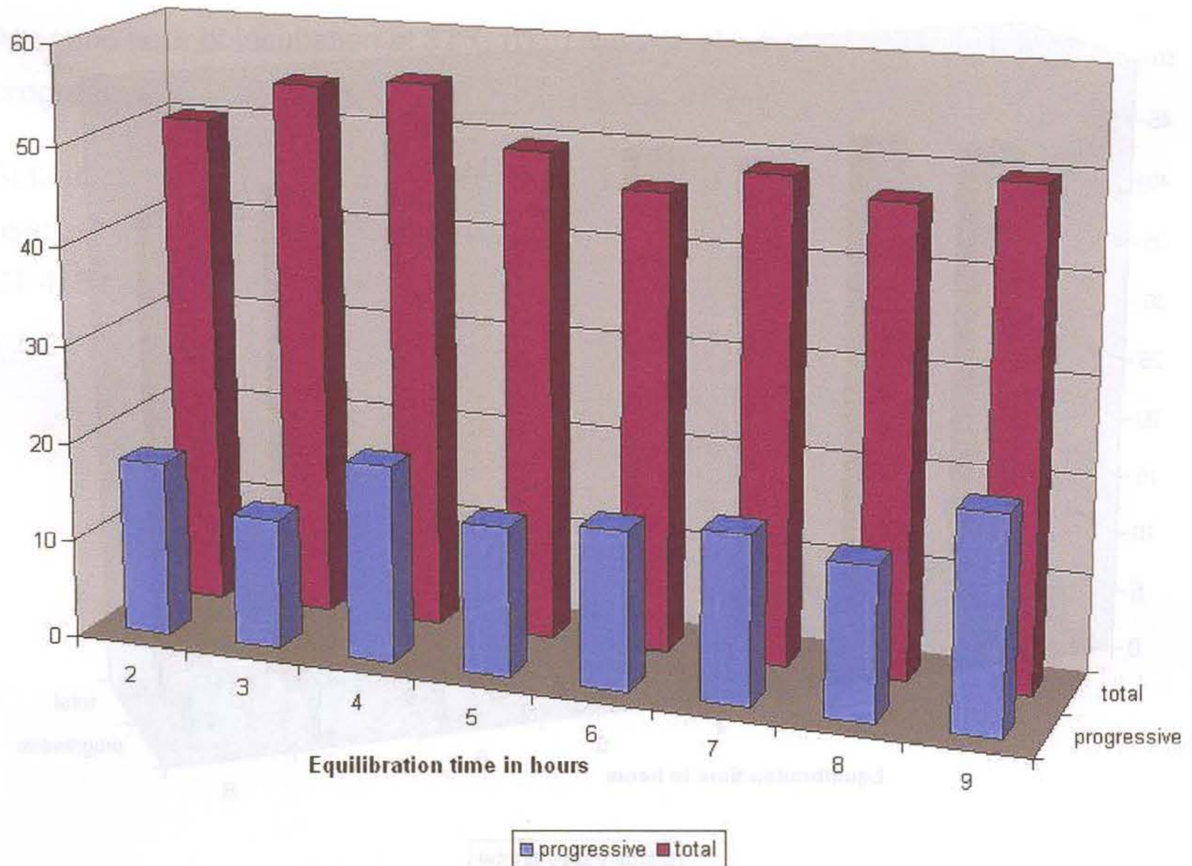

Figure 3.5 Progressive and total motility for epididymal sperm frozen with Triladyl two hours after thawing (mean values of samples taken from 11 buffaloes)

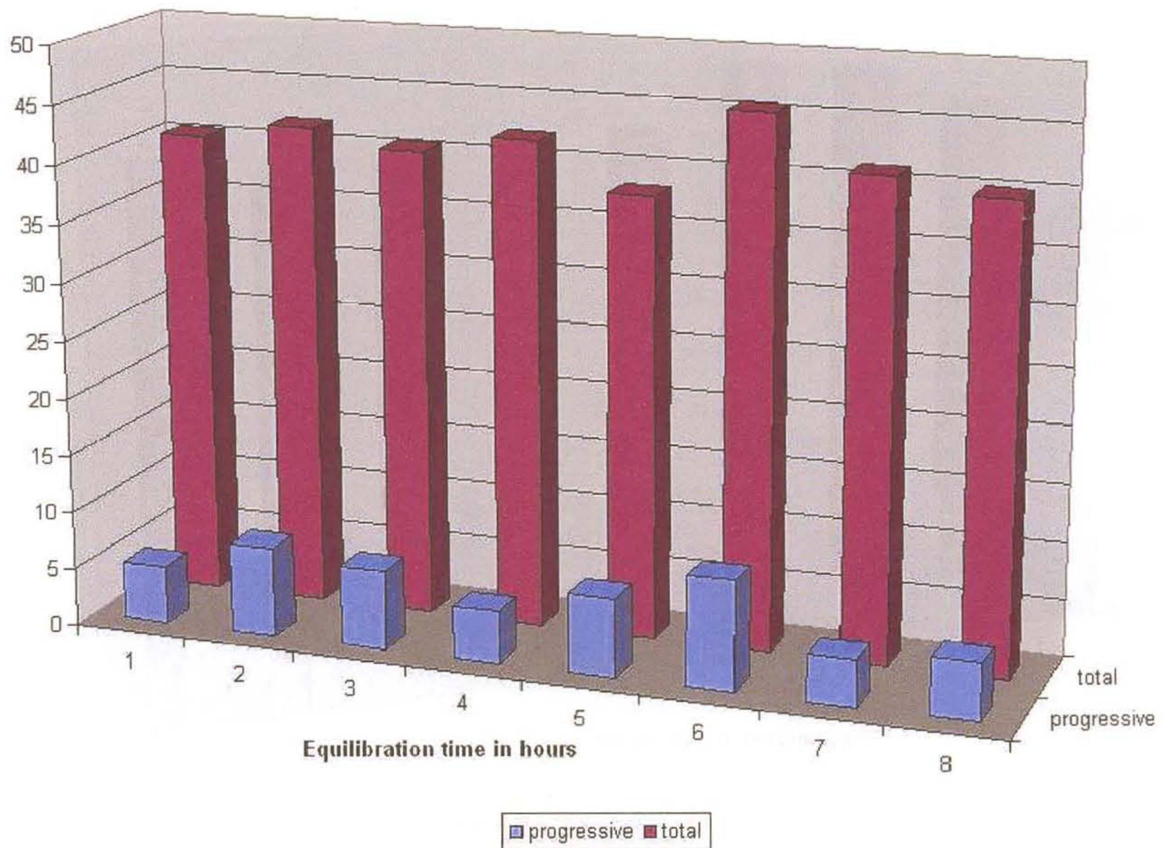

Figure 3.6 Progressive and total motility for epididymal sperm frozen with AndroMed two hours after thawing (mean values of samples taken from 11 buffaloes)

When AndroMed® and Triladyl™ were compared for each ET at t0, t1 and t2 Triladyl™ yielded almost always higher motilities. The differences were however not always significant. (see Table 3.1 and FiguresFigure 3.8Figure 3.9).

Immediately after thawing (t0) the use of Triladyl™ resulted in significantly higher total motility for ET 9. Samples frozen with Triladyl™ also showed significantly higher progressive motilities at t0 for ET 4.

After one hour of incubation at 37°C (t1) Triladyl™ showed significantly higher progressive motility for ET 2, ET 3, ET 4, ET 6 and ET 9 h.

At t2 total motilities for ET 2 and ET 4 were significantly higher when Triladyl™ was used. Progressive motility was significantly higher for the use of Triladyl™ at ET 2, ET 4, ET 5, ET 6, ET 8 and ET 9.

Table 3.1 Comparison of Triladyl™ (T) and AndroMed® (A) when the same equilibration times were used to freeze epididymal sperm (figures are mean values of samples taken from 11 buffaloes). Tot.0, Tot.1 and Tot.2 = total motility (in % ± SD) immediately, one and two hours after thawing respectively. Pr.0, Pr.1, Pr.2 = progressive motility (in % ± SD) immediately, one and two hours after thawing respectively. Differences are marked with asterisks when significant (* when $p<0.05$, ** when $p<0.01$ and * when $p<0.001$).**

	Medium	Tot.0	Pr.0	Tot.1	Pr.1	Tot.2	Pr.2
	T	50 ± 12	10 ± 8	56 ± 14	21 ± 15	50 ± 8	18 ± 11
2h	A	46 ± 9	11 ± 10	46 ± 11	11 ± 10	41 ± 10	5 ± 7
					*	**	**
	T	50 ± 16	18 ± 12	48 ± 15	20 ± 14	55 ± 16	13 ± 10
3h	A	44 ± 17	14 ± 15	44 ± 13	8 ± 10	42 ± 13	8 ± 9
					*		
	T	59 ± 8	23 ± 8	52 ± 14	23 ± 18	56 ± 14	20 ± 15
4h	A	51 ± 14	12 ± 13	47 ± 14	10 ± 10	41 ± 14	7 ± 9
			*		*	*	*
	T	54 ± 12	16 ± 11	58 ± 11	20 ± 15	50 ± 8	15 ± 11
5h	A	42 ± 20	14 ± 14	52 ± 21	10 ± 13	42 ± 18	5 ± 9
							*
	T	52 ± 13	23 ± 12	53 ± 14	23 ± 14	46 ± 10	16 ± 9
6h	A	48 ± 13	15 ± 17	44 ± 12	12 ± 10	38 ± 15	7 ± 7
					*		***
	T	55 ± 12	17 ± 11	54 ± 11	24 ± 7	49 ± 10	17 ± 8
7h	A	45 ± 14	19 ± 13	50 ± 17	13 ± 17	46 ± 13	10 ± 13
	T	49 ± 13	15 ± 14	51 ± 8	22 ± 13	47± 13	16 ± 11
8h	A	43 ± 11	14 ± 13	47 ± 22	13 ± 18	41 ± 18	4 ± 9
							*
	T	59 ± 11	18 ± 12	55 ± 13	24 ± 14	50 ± 9	22 ± 12
9h	A	47 ± 13	13 ± 11	46 ± 15	10 ± 12	40 ± 14	5 ± 8
		*			*		**

Equilibration time did not significantly affect post-thaw motility. Therefore the mean values of all equilibration times of each buffalo were used for further analysis. Triladyl™ always showed higher means for progressive motility at t0, t1 and t2 being always significant except for progressive motility immediately after thawing (t0) (see Table 3.2 and Figure 3.7).

Table 3.2 Comparison of Triladyl™ (T) and AndroMed® (A) irrespective of the equilibration times used to freeze epididymal sperm (figures are mean values of samples taken from 11 buffaloes). Tot.fr.,Tot.0, Tot.1 and Tot.2 = total motility (in % ± SD) of fresh sperm and immediately, one and two hours after thawing respectively. Pr.fr., Pr.0, Pr.1, Pr.2 = progressive motility (in % ± SD) of fresh sperm and immediately, one and two hours after thawing respectively. Differences are marked with asterisks when significant (* when $p<0.05$, ** when $p<0.01$ and * when $p<0.001$).**

Medium	Tot.fr.	Prog.fr	Tot.0	Pr.0	Tot.1	Pr.1	Tot.2	Pr.2
T	61 ± 15	21 ± 9	53 ± 12	17 ± 11	53 ± 13	22 ± 14	50 ± 11	17 ± 11
A	58 ± 17	35 ± 21	46 ± 14	14 ± 13	47 ± 16	11 ± 12	41 ± 14	6 ± 9
P			***		**	***	***	***

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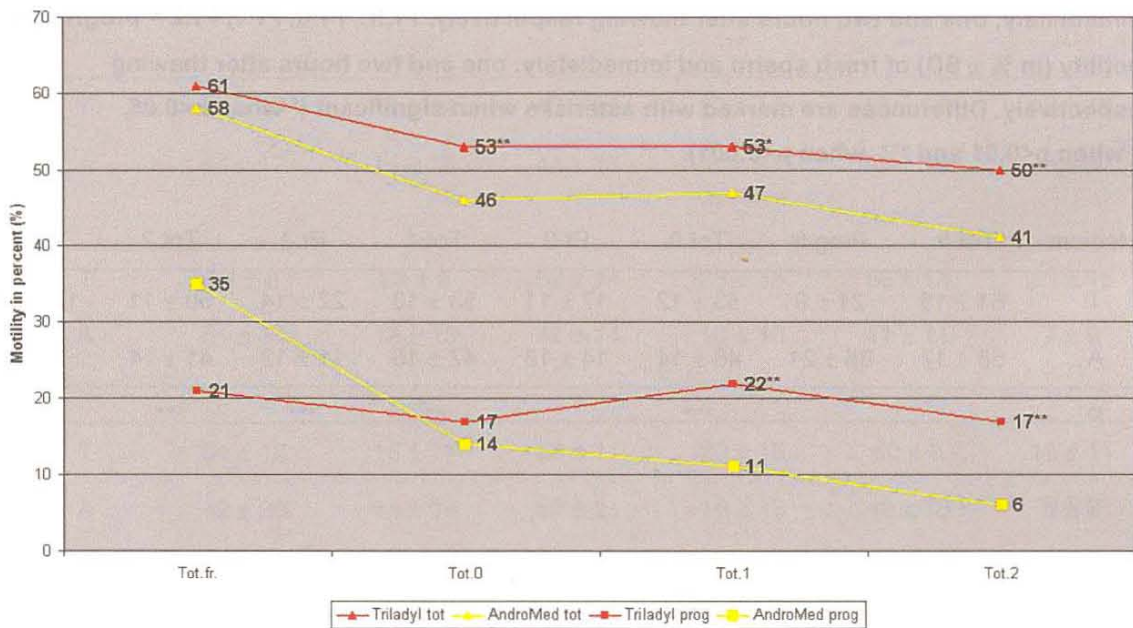

Figure 3.7 Comparison of longevity in respect of total and progressive motility for epididymal sperm frozen with AndroMed® and Triladyl™ (figures are mean values of samples taken from 11 buffaloes). Percentages marked with asterisks differ significantly (* = *P*<0.01, ** = *P*<0.001)

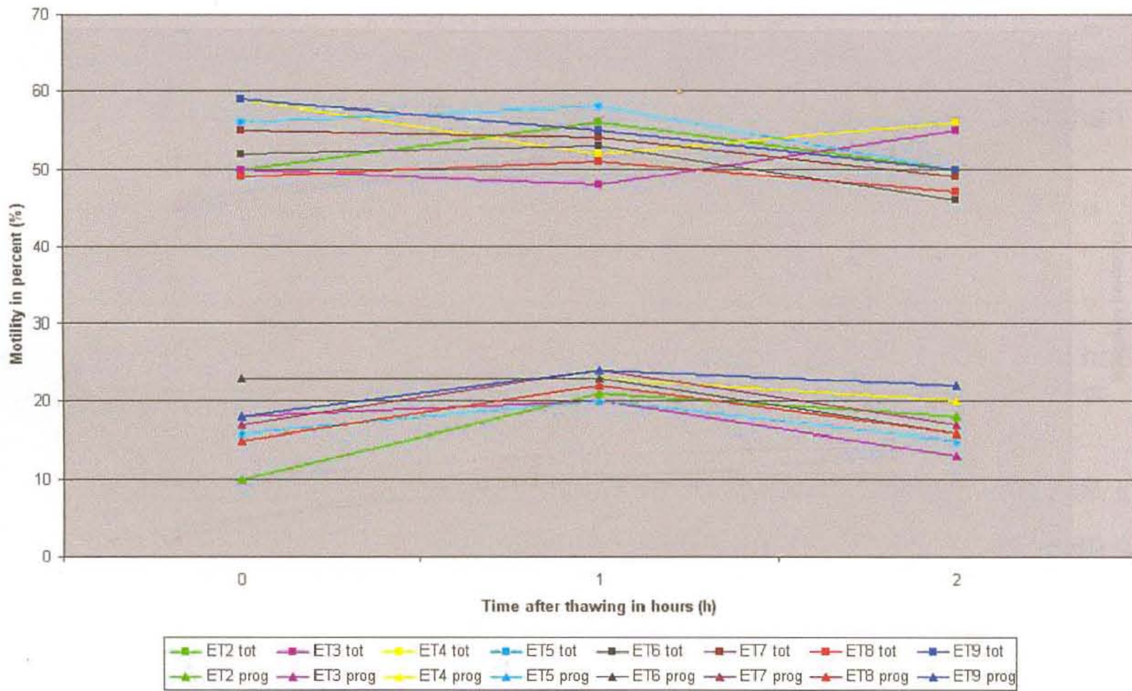

Figure 3.8 Comparison of total (tot) and progressive (prog) motility (%) in respect of different pre-freezing equilibration times (2-9h, ET2-ET9) for epididymal sperm frozen with Triladyl™ (figures are mean values of samples taken from 11 buffaloes)

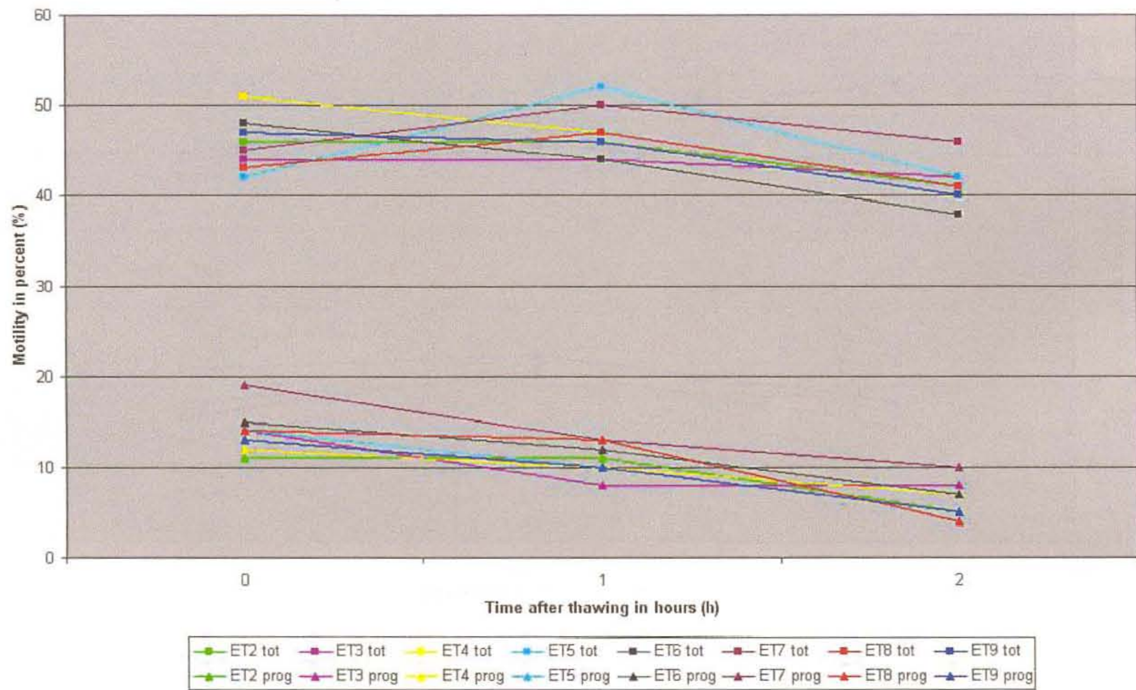

Figure 3.9 Comparison of total (tot) and progressive (prog) motility (%) in respect of different pre-freezing equilibration times (2-9h, ET2-ET9) for epididymal sperm frozen with AndroMed® (figures are mean values of samples taken from 11 buffaloes)

As ET had no influence on acrosomal integrity the mean values of all ET's from each buffalo were used to compare AndroMed® and Triladyl™. The use of Triladyl™ resulted in 56 ± 6 % intact acrosomes. This was significantly higher (P<0.05) than the results recorded for the use of AndroMed® (54. ± 6 %).

When lost acrosomes were compared an equilibration time of 6 hr resulted in significantly higher values than 4 or 8 hr (7 %, 4 % and 4 % respectively) using Triladyl™. Furthermore, the use of Triladyl™ resulted in significantly higher percentages (7 %) of lost acrosomes than AndroMed® (5 %), when equilibrated for six hours. AndroMed yielded significantly higher numbers of lost acrosomes after an equilibration time of 8 hours (7% and 4% respectively).

Total and progressive motility did not significantly decline within two hours after thawing. This was the case for both media. The progressive motility for samples frozen with AndroMed® after two hours were nevertheless less than half of what had been recorded immediately after thawing (6% and 14% respectively).

Total motility was significantly lower at t0 and t2 (46 % and 41 % respectively) than it was before freezing (58 %), using AndroMed®. Total motility did not differ between any time of evaluation when Triladyl™ was used.

Progressive motility was significantly lower at t0, t1 and t2 (14 %, 11 % and 6 % respectively) than it was before freezing (35 %), using AndroMed®. Progressive motility did not differ between any time of evaluation when Triladyl™ was used.

4 DISCUSSION

After decades of hunting and unscrupulous interfering in natural habitats man has finally realized the importance of preserving natural habitats and the diversity of species. Assisted reproductive techniques can be a useful tool in restoring the numbers of wild animals that have been threatened to extinction by humankind around the globe.

The collection and preservation of genetic material of wild animals can ensure the protection of a wide genetic diversity within a population even when numbers might be small at times after unforeseen incidences. There were such events in the past such as the outbreak of Rinderpest, which reduced most populations of cloven-hoofed animals in South Africa to small numbers (De Vos 1987).

Nowadays the numbers of African buffaloes are very favourable again, but the possibility of reintroducing them to former habitats is limited. This is due to the introduction of exotic diseases, which are now endemic in certain areas of South Africa such as the Kruger National Park. The possible transmission of foot-and-mouth disease, corridor disease, tuberculosis and brucellosis limits the transport of buffaloes and therefore also minimizes the possibility of increasing the genetic variety outside these endemic areas. Assisted reproductive techniques such as artificial insemination (AI) and in-vitro fertilization (IVF) might enable the use of genetic material of "diseased" buffalo populations without transmitting the actual diseases.

In this study we compared two different semen extenders according to their abilities to preserve epididymal sperm during freezing and thawing, in respect of resulting motilities and acrosomal integrities. Triladyl™ is a widely used medium, which has been shown to be suitable for the freezing of epididymal African buffalo sperm. The reasons for comparing this to a new medium are the possible risks of disease transmission and variable hygienic standards that are inevitable when egg yolk containing semen extenders are used. AndroMed® is a totally defined medium free of animal products and has already been used to freeze epididymal sperm from the African buffalo (Herold *et al.* 2003a).

When semen extenders that are free of animal products were compared to conventional ones in the past no differences have been found in the post thaw-total motilites (Bousseau *et al.* 1998; Müller-Schlösser *et al.* 2001 and Müller-Schlösser *et al.* 1995). These studies were however conducted on ejaculated bull semen and must therefore cautiously be compared to our results. In our study most differences became only obvious during evaluation of the longevity, which has not been recorded in the previously mentioned trials.

Results of a previous study performed in our laboratory indicate that sperm frozen with Trilady™ has a better longevity than when AndroMed® is used (Herold *et al.* 2003b). The results of this trial can only confirm these findings. We observed hardly any significant differences between the two media immediately after thawing and only a few after one hour of storage after thawing. Two hours after thawing samples treated with Triladyl™ showed significantly higher motilities for 6 out of 8 different equilibration times used.

Hinsch *et al.* (1997) found no differences total and linear motility and non-return rates, when Triladyl™ was compared to Biociphos®. There were no significant differences in the total and linear motility over the 8 hour period of the trial. At 5 and 8 hours after thawing, the linear motility tended to be higher for semen frozen with Biociphos® (54 and 53%) than for semen frozen with Triladyl™ (28 and 26%) ($p<0.6$ and 0.7). In our present study also most of the differences became only obvious 2 hr after thawing. Whether an increased longevity has an influence on the non-return rate needs to be tested. During routine inseminations many cows are not inseminated at the optimal time and it would therefore not be surprising if longevity has a significant effect on non-return rates in field trials. We also compared the motility values before freezing, immediately, one and two hours after thawing amongst each other. Only the use of Triladyl™ resulted in post-thaw motilities as high as fresh ones. The total as well as the progressive motility was on the other hand lower even right after thawing, than it had been before freezing, when AndroMed® was used. Although not significant an obvious decrease over time in progressive motility after thawing could only be seen when AndroMed® was used. The values immediately and two hours after thawing were 14 ± 13 and 6 ± 9 respectively. Whereas the

progressive motilities immediately and two hours after thawing for the use of Triladyl™ were 17 ± 11 and 17 ± 11 respectively.

Results of previous studies did not show differences in acrosomal integritiy when Triladyl™ was compared to the egg yolk-free medium Biociphos® (Hinsch *et al.* 1997). We found significant differences for lost acrosomes, but considered these differences to be neglect able for two reasons. Firstly because of the low numbers recorded (between 0 and 12) and secondly because of the fact that neither intact nor damaged acrosome values differed significantly for the comparison of the same treatments.

We did neither find differences among equilibration times for intact or damaged acrosomes nor for the comparison of the media for the same equilibration times each. We found nevertheless Triladyl™ to result in superior numbers of intact acrosomes when equilibration times were ignored.

Since a high motility rate is one of the prerequisites for successful artificial insemination it must be concluded that Triladyl™ is superior to AndroMed®. Even though the risks of egg yolk-containing diluents are known, they have, to the knowledge of the author, never resulted in spreading of disease when Specific Pathogen Free eggs were used. As I believe the advantages of higher motilities to be bigger than the hygiene risks I must conclude with the suggestion to use Triladyl™ rather than AndroMed®.

Another aim of this study was to establish the influence of exposure time of sperm to semen extender before freezing. Furthermore I wanted to establish the ideal equilibration time for both media, if any difference was to be found.

When ejaculated bull semen was used it has been suggested in previous studies that sperm survival is the higher, the shorter the exposure time to glycerol is (Berndtson and Foote 1969 and O´Dell and Hurst 1956). Contrary to these reports are results that indicate no differences in the percentage of intact acrosomes and motile spermatozoa in bovine semen samples when equilibrated for 0.5 or 2 hours (Wiggin and Almquist 1975a).

Our results are similar to the latter mentioned, even though it can't be said that they confirm these previous findings, as we used epididymal rather than ejaculated sperm and we used equilibration periods for up to 9 hours and buffalo sperm has been used in our study. There were nevertheless no differences for neither acrosomal integrity nor motility for equilibration times between 2 and 9 hours. This was the case for both media used.

In previous trials epididymal sperm from the African buffalo has always been cooled and stored within the whole epididymis. In our experiment we flushed the sperm first and only then cooled, froze and stored it. Results of these before mentioned trials nevertheless show that epididymal sperm can resist temperatures of around 4°C and motility can be restored thereafter. Friedmann *et al.* (2000) report that epididymal sperm from the African buffalo can maintain motility when stored for up to 24 hours at 4°C. Similar results of another study show that when motility was measured every 12 hours during storage a significant decline could only be seen after 36 h (Kilian *et al.* 2000). Another study did not only confirm that there were no differences to the initial motility after a storage time of 24 h, it has also been shown there that similar post-thaw motilities could be achieved after 24 h of exposure to 4°C when compared to sperm frozen immediately (Gerber *et al.* 2001). Contrary to these findings a significant decline in motility within the first 8 h has also been reported when epididymides were treated the same way (Bezuidenhout *et al.* 1995).

As stated above these results must only be compared very cautiously to ours since whole epididymides were cooled then and flushed sperm was frozen and stored in liquid nitrogenin our experiment now.

It seems nevertheless apparent that epididymal sperm from the African buffalo is capable to survive at least up to 8 h at temperatures around 4°C and their motility can be restored.

This is important to know since sperm collection in this species is always done in the field and precludes ideal laboratory conditions.

Because a high rate of progressive motile spermatozoa is one of the prerequisites for successful AI it must be concluded that Triladyl™ is superior to AndroMed®. As I believe the advantages of higher motility to be bigger than the hygiene risks I

conclude that epididymal buffalo sperm should rather be frozen with Triladyl™ than with AndroMed®. I can further conclude from the presented results that any equilibration time between 2 and 9 hr is suitable to freeze epididymal sperm from the African buffalo.

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