Preservation of fresh bovine semen and utero-tubal junction insemination in cattle

Steven Verberckmoes

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Dankwoord

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<tr>
<td>ABS</td>
<td>acryl butyl styreen</td>
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<tr>
<td>AI</td>
<td>artificial insemination</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CASA</td>
<td>computer assisted sperm analysis</td>
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<td>CD</td>
<td>conventional device</td>
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<tr>
<td>CEP</td>
<td>cauda epididymal plasma</td>
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<tr>
<td>CV</td>
<td>coefficient of variance</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GD</td>
<td>Ghent device</td>
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<tr>
<td>Hepes</td>
<td>N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Holstein Friesian</td>
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<tr>
<td>HHA</td>
<td>head to head agglutination</td>
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<tr>
<td>hpi</td>
<td>hours post insemination</td>
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<tr>
<td>ICSI</td>
<td>intracytoplasmatic sperm injection</td>
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<td>IUI</td>
<td>intra-uterine insemination</td>
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<td>IVF</td>
<td>in vitro fertilization</td>
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<tr>
<td>IVM</td>
<td>in vitro maturation</td>
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<tr>
<td>JC-1</td>
<td>5,5’,6,6’-tetrachloro-1,1’13,3’-tetraethylbenzimidazolyl carbocyanine iodide</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<td>LIN</td>
<td>linearity</td>
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<td>MC</td>
<td>methyl cellulose</td>
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<td>NRR</td>
<td>non-return rate</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>PMS</td>
<td>progressively motile spermatozoa</td>
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<td>PR</td>
<td>pregnancy rate</td>
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<tr>
<td>PSA</td>
<td>pisum sativum agglutinin</td>
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<tr>
<td>PVC</td>
<td>polyvinyl chloride</td>
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<tr>
<td>r</td>
<td>correlation coefficient</td>
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<td>RBC</td>
<td>red blood cells</td>
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<td>RMS</td>
<td>rapid progressively motile spermatozoa</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SQA</td>
<td>sperm quality analyser</td>
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<td>SST</td>
<td>sperm storage tubules</td>
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<tr>
<td>STR</td>
<td>straightness</td>
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<tr>
<td>TALP</td>
<td>tyrode solution supplemented with albumin, lactate and pyruvate</td>
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<tr>
<td>TCM</td>
<td>tissue culture medium</td>
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<tr>
<td>UTJ</td>
<td>utero-tubal junction</td>
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<tr>
<td>VAP</td>
<td>velocity average pathway</td>
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<tr>
<td>VCL</td>
<td>velocity curved line</td>
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<tr>
<td>VSL</td>
<td>velocity straight line</td>
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CHAPTER 1:

INTRODUCTION

1.1 State of the art: artificial insemination in cattle
1.2 Aims of the study
1.1 State of the art: artificial insemination in cattle

Artificial insemination (AI) or introduction of semen in the female genital tract by means of instruments is the first generation of reproductive biotechnologies which was feasible in cattle. The first commercial AI cooperative was established in 1936 by a Dane, Sorenson (Foote, 2002). Before the Second World War, most cows in Europe and North America were fertilized by means of natural service. However, since several cows on different farms were mated by the same bull, the spread of genital diseases with decreased fertility outcomes was a constant threat. Moreover, keeping herd bulls was expensive and represented potential danger for the herd manager (Vishwanath, 2003). Apart from these facts, the limited number of offspring produced per bull after natural mating made it impossible to set up effective progeny testing schemes and resulted in a very poor genetic gain. The introduction of AI in cattle was mainly forced by sanitary reasons, and especially by fertility problems caused by Campylobacter foetus subspecies venerealis (vibriosis) and Trichomonas foetus. However, also the control and prevention of non-sexually transmitted diseases such as tuberculosis, brucellosis and paratuberculosis at the farms benefited from the introduction of AI (Thibier and Guerin, 2000). Later on, the economic advantage by improved fertility rates and accelerated genetic progress became the most important motive (Foote et al., 1956; Watson, 1990).

In most developed countries, AI was introduced on a small scale during the 1940s and 1950s and was carried out with fresh semen or semen stored at room temperature. Notwithstanding the fact that satisfying pregnancy rates can be obtained with low doses of fresh semen, the high sire utilisation, the inexpensive sperm storage, and the easy use in the field, the use of fresh semen was seriously restricted by its limited shelf live. In the 1950s the beneficial effect of glycerol as cryoprotectant in diluents for freezing bovine semen was discovered (Polge and Rowson, 1952) and further optimization of the freezing-thawing procedure resulted in the wide spread application of frozen-thawed semen in the 1960s. The use of frozen-thawed semen facilitated the fast distribution of highly valuable genes, enabled the development of breeding programmes, and became most successful in the dairy cattle industry (Chupin and Schuh, 1993). An update survey in 1995 showed that the total number of doses of semen produced in the developed countries exceeded 200 million with more than 95% of this semen processed as a frozen product (Chupin and Thibier, 1995). The current world statistics for AI in cattle stand at 232 million doses of semen produced as a frozen
product and 11.6 million as liquid, with the latter restricted primarily to New Zealand with smaller amounts used in Africa, France, Australia, Germany, and Eastern Europe (Thibier and Wagner, 2000).

A recent interesting development in the assisted reproduction of cattle is the possibility to sort X- and Y-bearing spermatozoa by means of flowcytometry (Seidel, 2003). After sorting semen, the probability to have a calf of the desired gender after conception is 90%. This is in contrast to the 51% chance to have a bull calf after conception when non-sorted semen is used (Seidel, 2003). The possibility to determine the sex of the offspring before conception could be interesting for the dairy cattle industry. In dairy cattle, only a few bulls which highly inherit top productivity characteristics are needed for reproductive purposes. The other males are unprofitable and not wanted. Therefore, the more females are born, the better selection for productive and reproductive characteristics can be performed and the more milk can be produced at a farm. The use of sex sorted semen in dairy cattle could also result in a reduction of dystocia in heifers, and the opportunity to create an extra income out of the production of cross-bred beef bulls out of cows which are not good enough for production of replacement heifers. This means that more milk and meat could be produced with fewer animals, which is an extra advantage for the European dairy farmers, whose production is limited by the number of animals they may breed to comply with the law concerning environmental protection.

In Europe and the US, sex-sorted bovine semen is already a few years commercially available, however not widely used. Due to the high costs of the sexing procedure, a dose of sexed semen contains 1 to 2 million frozen-thawed spermatozoa, which is only 10% of a conventional insemination dose (Seidel, 2003). Moreover, the sexing procedure has a negative effect on sperm quality, which results in lower fertility rates (Seidel, 2003).

To improve fertility rates with low doses and less fertile semen (i.e. sex-sorted semen), two solutions are possible; 1) insemination with fresh instead of frozen-thawed semen, and 2) semen deposition closer to the site of fertilization.

The use of fresh semen for AI in cattle enables a ten fold reduction of the insemination dose compared to frozen-thawed semen without a reduction in pregnancy rates (Vishwanath et al., 1996). The freezing and thawing process has an irreversible impact on the spermatozoa, both on the recovery of motile, morphologically normal cells and the ensuing pregnancy rates (Holt, 2000). Bovine semen is the least sensitive of all species to freezing damages, but even with the best preservation techniques, the optimal cell recovery is just over 50% (Vishwanath
and Shannon, 2000). Many extenders have been developed for liquid storage of semen but, up to now, none of them is capable of storing spermatozoa for more than 3 days without a drop in in vivo fertility (Foote, 1978). Development of a diluent which could store semen for 4 to 5 days would be interesting for the cattle breeding industry for several reasons; 1) more insemination doses could be produced per ejaculate, 2) the work load during sperm collection and processing would be decreased, and 3) the distribution of liquid semen would be simplified.

When conventional insemination is performed in cattle, 10 to 15 million frozen-thawed spermatozoa are deposited into the uterine body, while fertilization occurs in the tuba uterine (oviduct). Compared to conventional insemination, semen deposition near the utero-tubal junction could theoretically be performed with 100-times less spermatozoa without a decrease in fertility due to the reduced loss of spermatozoa by 1) retrograde flow in the cervical mucus (Larsson and Larsson, 1985; Mitchell et al., 1985; Nelson et al., 1987), 2) decreased phagocytosis during migration through the uterus (Hawk, 1983), and 3) an increase in survival time of spermatozoa in the sperm-friendly environment of the isthmus (Suarez, 2001).
1.2 Aims of the study

To increase the number of insemination doses produced by genetically highly valuable sire bulls, the use of liquid-stored semen instead of frozen-thawed semen and deposition of semen near the utero-tubal junction (UTJ) instead of into the uterine body, could be appropriate alternatives.

Research is needed to increase the time during which semen can be stored in liquid state whilst maintaining its fertilizing capacity. Since bovine spermatozoa can be stored for several weeks in the cauda epididymidis without negative effects on their fertilizing capacity, the cauda epididymal fluid can serve as basis for the development of a sperm diluent to prolong the shelf life of freshly ejaculated bovine semen. In order to deposit semen near the UTJ, a special insemination device has to be developed which has no detrimental effect on the sperm quality and on the endometrium of the cow in oestrus, and which can easily be handled by one person under field conditions.

In order to achieve these aims, the study comprises the following experiments:

1. Determination of the composition of the cauda epididymal plasma (CEP) of the bull
2. Development of a new diluent (CEP diluent) based on the ionic composition of the bovine cauda epididymal plasma
3. Comparison of the storage capacity of the CEP diluent with two other diluents for extended preservation of liquid bovine semen
4. Assessment of a sperm migration assay in a synthetic medium and its relation to in vivo bull fertility
5. Development of a new insemination device (Ghent device) for utero-tubal junction insemination in dairy cattle
6. Assessment of the Ghent device for utero-tubal junction insemination in dairy cattle with full insemination doses
7. Assessment of the Ghent device for utero-tubal junction insemination in dairy cattle with reduced insemination doses
8. Effect of whole blood and serum on bovine sperm quality and in vitro fertilization capacity
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CHAPTER 2

INTRA-UTERINE INSEMINATION IN FARM ANIMALS AND HUMANS

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Verberckmoes S., Van Soom A., de Kruif A.
ABSTRACT

Artificial insemination (AI) is the oldest and currently most common technique in the assisted reproduction of animals and humans. The introduction of AI in farm animals was forced by sanitary reasons and the first large scale applications with a commercial goal were performed in cattle in the late thirties of last century. After the Second World War, cryopreservation of semen facilitated distribution and AI was mainly performed for economic reasons, especially in dairy cattle industry. In humans however, AI was originally performed in cases of physiological and psychological sexual dysfunction, but later on also in cases of infertility caused by immunological problems. Currently, the most common indications for intra-uterine insemination (IUI) in humans are idiopathic infertility and male subfertility. In these cases, IUI is considered as the treatment of the first choice, before more invasive techniques such as in vitro fertilization (IVF) and intracytoplasmatic sperm injection (ICSI) are used. In contrast with humans, the quantity and quality of spermatozoa produced by farm animals is much higher and permits dilution and production of several insemination doses per ejaculate. However, with the introduction of sex-sorted semen in farm animals, the same problem of low quality semen as in humans has arisen. In cattle, pigs and horses, conventional insemination with low numbers of sex-sorted spermatozoa results in a significant decrease in fertility. To improve the fertility rates with this semen, new insemination techniques have been developed in order to deposit spermatozoa closer to the site of fertilization. In sows and mares the advantage of utero-tubal junction insemination has already been proven, however in cattle it is still under investigation. In this review, the differences and similarities in the application of AI between animals and humans are discussed and since AI in farm animals is most successful in cattle, the situation in this species is elaborated the most.
INTRODUCTION

Artificial insemination (AI) or introduction of semen in the female genital tract by means of instruments is the first generation of reproductive biotechnologies which was feasible in farm animals, both historically and in terms of numbers around the world (Chupin and Thibier, 1995). The first report of artificial insemination (AI) in animals was made by the Arabs and dates from around 1322. Semen of Arabian stallions was collected and used for fertilization of mares; however the mechanism of fertilization was still unknown at that time. The first steps in andrology were made by Van Leeuwenhoek in 1678 (Foote, 2002), who developed a microscope for sperm observation. He was the first man who could visualise spermatozoa and called them “animalcules”. About 4 centuries after the Arabs, Spallanzani (1784), in Italy, discovered that a bitch could be impregnated with the cellular portion of semen. He also demonstrated that cooling and freezing could inactivate spermatozoa, while they could be reactivated by warming. Around 1900, Professor Ivanoff was hired by the Russian throne to develop AI in horses. By 1922 he had developed methods for collecting semen and inseminating horses, cattle, sheep and swine (Ivanoff, 1922). Semen of stallions was collected by insertion of a sponge into the vagina of the mare before natural mating, and the sponge was subsequently used for insemination of other mares. Much of Ivanoff’s work was taken over by Milovanov (1938), who designed artificial vaginas and other items, many similar to those used today. The first commercial AI cooperative was established in 1936 by a Dane, Sørenson (Foote, 2002). First, AI in cattle was performed with fresh semen but since 1965 predominantly frozen-thawed semen is used. Currently, AI is performed in all farm animals: cattle, horses, sheep, goats, pigs, chickens, turkeys, rabbits, bees, …. However, the technical advancement in AI has been captured most successfully in dairy cattle (Vishwanath, 2003). During the last decade, the use of AI in swine production has increased dramatically from about 5% of all matings in 1990 to about 60 to 70% in 2000 in developed countries (Singleton, 2001; Vyt et al., 2004). However, in contrast to cattle, frozen-thawed semen is rarely used in pigs (Singleton, 2001; Vyt et al., 2004).

Originally, AI in farm animals was introduced for sanitary reasons. Later on, the economic advantage which could be gained by improved fertility rates and accelerated genetically progress became the most important motive (Foote et al., 1956; Watson, 1990).
Lowering the insemination dose, without a decrease in fertility rate, would increase the number of inseminations and descendants of highly valuable sires. This would not only be advantageous for the AI centres but also for farmers.

In humans, AI has been used in clinical medicine for over 200 years in the treatment of infertile couples. The first documented application of AI was performed in London in the 1770s by John Hunter (Siegler, 1944). A patient with severe hypospadias was advised to collect the semen (which escaped during the coitus) in a warmed syringe and to inject the sample into the vagina. In 1873 Dr. Sims reported his findings on 55 inseminations. Intrauterine insemination in humans came into widespread clinical use in the 1960's.

Initially, unprepared sperm was used for IUI which sometimes led to serious side effects such as introduction of infection or painful uterine contractions provoked by seminal prostaglandins (Rowell and Braude, 2003). These problems were resolved by preparation of the semen and removal of the seminal plasma before insemination (Martinez et al., 1993). An alternative for IUI is in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI), followed by embryo transfer (Oehninger, 2001). A great step forward in human assisted reproduction was made by the introduction of ICSI (Palermo et al., 1992, 1996; Van Steirteghem et al., 1993). ICSI opened new perspectives in the treatment of extreme male subfertility and made it possible to use epididymal and testicular sperm (Silber et al., 1995) and even acrosomeless spermatozoa (Lundin et al., 1994). Currently, IVF with or without ICSI is frequently used in severe cases of infertility (Oehninger, 2001).

When artificial insemination is performed in animals and humans the cervical-mucus barrier is bypassed in order to increase the gamete density at the site of fertilization (Allen et al., 1985). This represents the common factor in AI applied in animals and humans. However, apart from this factor, there are not many similarities between human and animal AI. Not only do the anatomical differences interfere with the application, but more importantly the substantial difference in sperm quality between men and animals leads to totally different indications for AI. In this review, we will focus on the differences between farm animals and humans, as far as the indications, semen quality, and outcome of IUI are concerned.
INDICATIONS FOR AI IN ANIMALS AND HUMANS

In animals, AI was introduced mainly for sanitary reasons. Before the introduction of AI, cows on different farms were mated by the same bull, which frequently resulted in spreading of venereal diseases and concomitant fertility problems. In cattle, most of the fertility problems related to natural mating were due to *Campylobacter fetus* subspecies *venerealis* (vibriosis) and *Trichomonas foetus*, while *Taylorella equigenitalis* was the most important infectious agent causing subfertility in mares (Hoffer, 1981; Timoney, 1996; de Kruif, 2003). Also, the control and prevention of non-sexually transmitted diseases such as tuberculosis, brucellosis and paratuberculosis benefited from the introduction of AI (Thibier and Guerin, 2000).

Another great advantage of AI in farm animals was the rapid dispersal of valuable genes and the ability to improve the genetic quality of the livestock, and the reduction of the number of lethal genes (Foote et al., 1956; Watson, 1990). The introduction of AI in the pig, poultry and rabbit industry resulted in a rapid improvement of the carcass quality, and enabled the expansion and specialization of breeding units (Singleton, 2001). In this way, the application of AI has become a feasible and cost effective technology in the intensive farm animal industry. In poultry, AI is almost exclusively performed in turkeys for commercial flock production (Donoghue and Wishart, 2000). The reason for this is the contrast in size between toms (large white strains can exceed 33 kg) and hens (approximately 9 kg at the onset of the lay) which hampers natural mating. Moreover the extreme selection for growth is related to a decrease in fertility, leading to an inevitable integration of AI in the commercial poultry production (Reddy, 1995; Donoghue and Wishart, 2000). However, the application of AI in farm animals also has disadvantages. Probably the most important disadvantage is the limited number of genetic highly valuable sires (mostly of the same family) which is used on a large scale. This increases the risk of inbreeding and concomitant genetic defects.

The introduction of glycerol as cryoprotectant for semen, and improvements in the freezing protocol, made it possible to inseminate livestock species with frozen-thawed semen. The use of frozen-thawed semen facilitated the fast distribution of valuable genes and the development of breeding programmes. The development of breeding programmes based on AI has been most successful in the dairy cattle industry (Chupin and Schuh, 1993;
Chupin and Thibier, 1995). Before a dairy bull is used as a sire, it has to pass several selection criteria. At first it is used as a “test bull”. During this period, semen is collected, evaluated and subsequently frozen. A few hundred cows are inseminated with this semen to determine 1) the fertility of the bull and 2) the production and conformation characteristics of the bull’s daughters. After three to four years, all data on non-return rates (fertility) of the test bull, and on production and conformation characteristics of its daughters, are analysed. At this moment, it is decided whether or not the bull can be used as sire on large scale for insemination purposes. The application of such breeding programmes decreases the number of bulls used for reproduction but largely increases the number of descendants of highly valuable bulls. However, freezing semen is not as successful in all farm animals as it is in cattle. Semen of horses, pigs, sheep, goats, poultry and rabbits is more susceptible to freezing damage. In these animals, fertility results with frozen-thawed semen are much lower than with fresh semen, unless special insemination techniques are used (Mocé et al., 2003; Armstrong and Evans, 1984; Salamon and Maxwell, 2000; Donoghue and Wishart, 2000; Johnson et al., 2000; Leboeuf et al., 2000; Linfor and Meyers, 2002).

In humans, AI was originally only performed in cases of physiological and psychological dysfunction, such as retrograde ejaculation, hypospadias impotence, and vaginismus. Afterwards AI was used for the treatment of male infertility due to immunological causes or uncorrectable semen deficiencies, for a desired pregnancy by single or lesbian women, or as an alternative source of semen during cycles of assisted reproductive technology when the original source of semen is unsuitable (Bronson et al., 1984; Barratt et al., 1992; Wolf et al., 2001). Nowadays, the most common indications for AI in humans are moderate male subfertility and unexplained infertility (Ombelet et al., 1995). According to the literature, AI can significantly increase the chance of conception in idiopathic and moderate male subfertility when compared with the estimated spontaneous pregnancy rate or pregnancy rate after timed intercourse (Cohlen et al., 2000). Two important studies in the Netherlands and the United Kingdom (Goverde et al., 2000; Philips et al., 2000) demonstrated that three cycles of intra-uterine insemination (IUI) offer the same cumulative ongoing pregnancy rate as in vitro fertilization (IVF), whilst being more cost-effective.
It is clear that the indications for IUI in humans and farm animals are quite different. IUI in humans is performed in cases of fertility problems, while AI in animals is performed for economic reasons. Moreover, both the higher natural fertility of cattle and the stringent selection criteria of the breeding programmes in animals result in a population of breeding sires which have a much higher semen quality than the average human sperm donor.

SEMEN QUALITY IN ANIMALS AND HUMANS

Farm animals which are selected for the production of meat or milk are held for commercial and not for emotional purposes. In such animals (cattle, swine, poultry, rabbits, sheep and goats), a rigorous selection for fertility has been performed in males leading to sires with outstanding semen quality. In animals which are kept as companion animals, such as dogs and horses, selection for fertility has not been as strict as for farm animals, which has led to a much higher variation in semen quality among stud dogs and stallions.

The same basic semen analysis is used in humans and animals: evaluation of volume, concentration, percentage of membrane intact spermatozoa, sperm morphology and percentage of total and progressively motile spermatozoa. The final goal of sperm assessment is to predict the fertilizing capacity. In cattle, several studies have already been performed to find a simple and reliable test to predict in vivo fertility (Larsson and Rodriguez-Martinez, 2000). Despite the fact that a number of studies have focused on single sperm traits such as sperm morphology (Barth, 1993), sperm motility (Stalhammar et al., 1994; Holt et al., 1997), and the presence of intact acrosomes (Cumming, 1995), none of these traits have been significantly correlated with the in vivo fertility of bulls. However, combination of the different sperm traits can increase the reliability of the prediction of the fertilizing capacity (Zhang et al., 1999; Amann and Hammerstedt, 1993; Farrell et al., 1998; Rodriguez-Martinez, 2003). Moreover, under certain conditions, a positive association can be found between the number of bovine spermatozoa bound to 0.1 mm² oviductal epithelium and in vivo fertility of bulls (De Pauw et al., 2002). Since this sperm-oviduct binding assay is new and can only be performed in a well equipped laboratory, it is not widely used. Up to now, determination of the progressive motility of the semen sample remains the most important factor to predict the in vivo fertility of the animal in question. However, since only normally to highly fertile sires are used for AI in
farm animals, the outstanding sperm quality in all of these sires makes it impossible to predict the relatively small differences in fertility outcomes based on a single sperm trait.

However, in other species such as dogs and horses, the variability in sperm quality and in vivo fertility is much higher than in farm animals (Loomis, 2001). In horses, a conventional breeding soundness examination is sufficient to identify stallions that clearly lack the capacity for adequate fertility. However, it does not mean that these stallions are excluded from breeding (Colenbrander et al., 2003). Just as in cattle, accurate prediction of the level of fertility and identification of some subfertile stallions is not always possible (Colenbrander et al., 2003). Like in horses, fertility is not a major selection criterion in dog breeding, and popular dogs with sperm of very poor quality can be used for breeding purposes without any restriction. However, in contrast to horses, the average sperm quality in dogs is very high (Sieger, 1986; Thomassen et al., 2001).

The method of sperm assessment in humans is very similar to that in animals and has similar limitations. With an exception for donor insemination, male fertility is not a limiting criterion in human reproduction. Semen of donors is only used if it is of good quality and free of infectious diseases (American Fertility Society, 1990). Compared to farm animals, the variation in semen quality in humans is much larger and, as already stated, the fertility of the man is much lower as well (Table 1). The huge variation in human sperm quality facilitates the prediction of the fertility by means of sperm traits. The use of strict morphology criteria (Tygerberg strict criteria) has been proven to be helpful in predicting IVF (Van Waart et al., 2001) and IUI outcomes (Coetzee et al., 1998). To achieve acceptable pregnancy rates after IUI, at least 5% of the spermatozoa have to be morphologically normal (Ombelet et al., 1997; Montanaro-Gauci et al., 2001; Van Waart et al., 2001). When normal sperm morphology exceeds 14%, the chance of pregnancy after IUI is 1.8 times higher than when it is lower than 14% (Montanaro Gauci et al., 2001). Apart from morphology, other parameters, particularly motility and insemination motile sperm count (IMC), have been shown to be useful in predicting the outcome of IUI. When sperm motility is higher than 50%, the chance of success after IUI is 2.95 times higher compared to <50% motility (Montanaro Gauci et al., 2001).
EFFECT OF FREEZING AND SEXING OF SEMEN ON FERTILITY RATES

Before 1950, most inseminations in cattle were performed with fresh semen. However, fresh semen has a limited shelf-life of only a few days, which seriously restricts the ease of distribution and use in distant locations (Vishwanath and Shannon, 2000). Many extenders have been developed for liquid storage of semen but, up to now, none of them is capable of storing spermatozoa for more than 3 days without a drop in in vivo fertility (Foote, 1978). The development of a diluent which could store semen for 4 to 5 days would solve the distribution problem and could increase the number of insemination doses that can be produced from one ejaculate (De Pauw et al., 2000).

As opposed to fresh semen, cryopreservation of semen is a mean of indefinite sperm storage. However the freezing and thawing process has an irreversible impact on the spermatozoa, both on the recovery of motile, morphologically normal cells and the ensuing pregnancy rates (Holt, 2000). Bovine semen is the least sensitive of all species to freezing damages, but even with the best preservation techniques, the optimal cell recovery is just over 50% (Vishwanath and Shannon, 2000). In cattle, similar pregnancy rates can be obtained with frozen-thawed spermatozoa and with fresh semen (55% per cycle), but insemination doses need to be 10 times higher when using the former (Shannon, 1978; Foote and Parks, 1993). Another advantage of frozen semen, besides the ease of distribution, is that it enables screening of the bull for infectious diseases before its semen is used in the field. Currently, more than 95% of the semen in cattle is processed as a frozen product (Chupin and Thibier, 1995), while the use of liquid semen is restricted primarily to New Zealand with smaller amounts used in Africa, France, Australia, Germany, and Eastern Europe (Thibier and Wagner, 2000). To maximize the number of insemination doses produced per sire bull, dilutions are made to determine the optimal number of spermatozoa per dose. In accordance with the number of spermatozoa required per insemination dose (5, 10 or 15 x 10^6 of frozen-thawed spermatozoa respectively) to achieve acceptable pregnancy rates, a classification can be made between highly, moderately and lowly fertile sire bulls (Den Daas et al., 1998). With an average production of 5 to 6 x 10^9 spermatozoa per ejaculate and an optimal frequency of 6 collections per week, a production of 30 to 40 x 10^9 spermatozoa per week can be reached. With a 50-week-per-year collection schedule and 10 x 10^6 frozen-thawed spermatozoa per
insemination dose, these sperm numbers can be translated in 200,000 doses of semen for AI, per bull, per year (Vishwanath, 2003). Due to the higher susceptibility of semen of other farm animals to freezing damage, the use of frozen-thawed semen is much less important and mostly not as cost effective as it is in cattle.

It is obvious that the situation is completely different in humans, in whom sperm quality is much less and in whom a whole ejaculate is required to achieve pregnancy (Table 1). Except for donor inseminations, fresh semen is used for insemination purposes. However, due to the poor semen quality, fresh as well as frozen sperm used for IUI is washed and prepared before use to improve fertilisation rates (Aitken and Clarkson, 1987). Washing procedures remove prostaglandins, infectious agents, antigenic proteins, non-motile spermatozoa, leukocytes and immature germ cells. To concentrate the number of motile spermatozoa, glass-wool filtration or a density gradient such as in a swim-up, Puresperm® centrifugation (NidaCon International AB, Gothenburg, Sweden) or Percoll® centrifugation (Pharmacia, Uppsala, Sweden) can be used (Erel et al., 2000; Sakkas et al., 2000; Tomlinson et al., 2001a,b). In the two latter techniques, cellular debris, non-motile spermatozoa, and abnormal spermatozoa are trapped at the interface, while motile spermatozoa with normal head morphology move to the bottom of the tube. In a swim-up, motile spermatozoa are gathered at the top of the tube. The average pregnancy yield in humans after IUI with 1 million selected normal motile spermatozoa is approximately 13% per cycle (Ombelet, 2003). This is much lower than the 55% pregnancy rate per cycle obtained after IUI in cattle with 10 million unselected frozen-thawed spermatozoa or with 2 million unselected fresh spermatozoa (Foote and Parks, 1993).

Table 1: Criteria applied for fresh semen of men (semen donors) and bulls considered for IUI.

<table>
<thead>
<tr>
<th></th>
<th>human</th>
<th>cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td>volume</td>
<td>≥ 2 ml</td>
<td>≥ 2 ml</td>
</tr>
<tr>
<td>concentration</td>
<td>≥ 50 x 10^6 /ml</td>
<td>≥ 600 x 10^6 /ml</td>
</tr>
<tr>
<td>total motility</td>
<td>≥ 50%</td>
<td>≥ 80%</td>
</tr>
<tr>
<td>progressive motility</td>
<td>≥ 25%</td>
<td>≥ 65% (≥ 35% after thawing)</td>
</tr>
<tr>
<td>normal morphology</td>
<td>≥ 8%</td>
<td>≥ 80%</td>
</tr>
<tr>
<td>total sperm count</td>
<td>≥ 40 x 10^6</td>
<td>≥ 1.2 x 10^9</td>
</tr>
<tr>
<td>white blood cells</td>
<td>≤ 1 x 10^6</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = not determined
In humans, the same criteria are used for semen donors as for fertility patients, with an exception for sperm concentration: $\geq 20 \times 10^6$/ml. Data adapted from WHO manual 1999 and A. de Kruif (personal communication).

Recently, however, a situation comparable to that in humans has arisen in farm animals. This is due to one of the most important technical advances in sperm processing: namely sexing of sperm by DNA quantification using flowcytometry. The ability to separate X- and Y-bearing spermatozoa could be of considerable economic advantage in the farm animal industry (Seidel, 2003). However, the limited number of spermatozoa that can be sorted per hour limits its extensive commercial application. Moreover, the low number of spermatozoa per insemination dose and the lower sperm quality obtained after the sexing process severely impairs fertility results obtained with sex-sorted semen. Published data about pregnancy rates obtained with sex-sorted semen in cattle are only available from heifers under experimental conditions. Pregnancy rates with sex-sorted semen were significantly lower than with unsexed semen (Table 2) (Seidel et al., 1999). Moreover, no difference was found between deposition of sex-sorted semen in the uterine body and uterine horns (Seidel et al., 1999). Since the size of the uterus of older cows is much larger than in heifers, more sperm loss can be expected and semen deposition near the UTJ could be advantageous. More research with sexed semen in breeding cows stressed by heavy lactation, and in superovulated cows, is currently under investigation.

Table 2: Pregnancy rates obtained with sex-sorted semen in the uterine body and horns of heifers.

<table>
<thead>
<tr>
<th>Treatment / site</th>
<th>No. of sperm</th>
<th>No. of heifers</th>
<th>No. pregnant d 60-63</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sexed, frozen / body</td>
<td>$1.5 \times 10^6$</td>
<td>27</td>
<td>9 (33%)$^a$</td>
</tr>
<tr>
<td>Sexed, frozen / body</td>
<td>$3.0 \times 10^6$</td>
<td>25</td>
<td>9 (36%)$^b$</td>
</tr>
<tr>
<td>Sexed, frozen / horns</td>
<td>$1.5 \times 10^6$</td>
<td>24</td>
<td>7 (29%)$^b$</td>
</tr>
<tr>
<td>Sexed, frozen / horns</td>
<td>$3.0 \times 10^6$</td>
<td>24</td>
<td>8 (33%)$^b$</td>
</tr>
<tr>
<td>Control, frozen / body</td>
<td>$20 \times 10^6$</td>
<td>24</td>
<td>17 (71%)$^b$</td>
</tr>
</tbody>
</table>

$^a,b$Means without common superscripts differ (P<0.05). From Seidel et al., 1999.

SPERM DISTRIBUTION AND POPULATION OF THE SPERM RESERVOIR

When cattle, sheep, goats and dogs are mated, semen is deposited in or in front of the cervix uteri. Before oocytes can be fertilized in the ampulla of the oviduct, spermatozoa have to pass the cervical barrier and migrate through the uterine body to populate the functional sperm reservoir in the isthmus of the oviduct (Suarez, 1999). During the
transport of the spermatozoa to the site of fertilization, the number of spermatozoa decreases tremendously (Hunter and Greve, 1998). A great deal is lost by retrograde flow in the cervical mucus (Larsson and Larsson, 1985; Mitchell et al., 1985; Nelson et al., 1987) and by phagocytosis during migration through the uterus (Hawk, 1983). Once the spermatozoa have passed the utero-tubal junction, they enter the isthmus of the oviduct, where a functional sperm reservoir is established (Hunter and Wilmut, 1982, 1984; Yanagimachi, 1994; Suarez, 1999). In this sperm friendly environment, spermatozoa are attached to the apical plasma membrane of the ciliated and secretory epithelial cells until the moment of ovulation (Suarez et al., 1990). In the isthmus, bovine spermatozoa can remain arrested for 18 hrs and only detach near the time of ovulation (Hunter and Wilmut, 1984). At the time of activation of the newly-ovulated oocyte, the sperm:egg ratio at the ampullary-isthmic junction may be close to 1:1 (Hunter and Greve, 1998).

Exceptional kinds of sperm reservoirs are present in poultry and bees and are called “fossulae spermaticae” and “spermateka” respectively. The fossulae spermaticae are found in the distal half of the oviduct of all avian species studied to date, sequester and store spermatozoa which are slowly released over time to insure an adequate population of spermatozoa at the site of fertilization (Bakst, 1993). However, in contrast to the sperm reservoir in mammalian farm animals, spermatozoa can be stored for a much longer period of time. Turkey hens for example, can produce fertilized eggs up to 16 weeks after insemination when inseminated before onset of egg production (Christensen and Bagley, 1989). The spermateka in bees has a similar function as the fossulae spermaticae in poultry, but spermatozoa can be stored in this reservoir for 3 to 4 years (Pechhacker, 2003). Due to the presence of these specific sperm reservoirs in poultry and bees the moment of insemination is of low importance.

In glaring contrast to poultry and bees, determination of the ideal moment of insemination in mammalian farm animals and humans is very important. In general, the ideal moment of insemination in cattle is at 12 hrs after onset of oestrus (Hunter and Greve, 1997). However, since detection of oestrus is not always easy, determination of the ideal insemination moment is hampered (Van Eerdenburg et al., 1996). In humans, IUI insemination is most frequently performed after controlled ovarian hyperstimulation (COH) and the moment of ovulation can be predicted rather well. Moreover, women can be monitored very well by means of ultrasound scanning, analysis of luteinizing hormone
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(LH), estradiol (E2), and progesterone (P) concentration to determine the best moment of insemination (Check et al., 1994; Filicori et al., 2003).

When conventional insemination is performed in cattle, horses and pigs, semen is deposited in the uterine body. In cattle, by-passing the cervix, which is the major barrier to sperm transport at natural mating, allows a 500-fold reduction of the insemination dose without a decrease in fertility rate (Senger, 1993). Conventional insemination in cattle is performed under rectal guidance with a stainless steel Cassou insemination device, which is covered by a plastic sheet to prevent damage to the female genital tract. In horses and pigs, IUI can be performed in a rather similar way as in cattle. In ewes, goats and dogs however, passage of the cervical folds is very difficult and best results for IUI are obtained with laparoscopy (Hill et al., 1998, Verberckmoes et al., 2001). An alternative for IUI in these species is intra-vaginal insemination. However at least 10 times as many spermatozoa are required to obtain similar pregnancy rates as by IUI (Tsutsui et al., 1988; Linde-Forsberg et al., 1999). Moreover, when intra-vaginal insemination in these species is performed with frozen-thawed semen, fertility rates decrease tremendously (Armstrong and Evans, 1984; Salamon and Maxwell, 2000; Thomassen et al., 2001).

In the fifties, a good deal of research in cattle was also performed to compare deep intra-uterine insemination (in uterine horns) with conventional insemination (in the uterine body) (Knight et al., 1951; Salisbury and VanDemark, 1951; Stewart and Melrose, 1952; Olds et al., 1953). However, in most of these studies, which were performed with high numbers of fresh semen, no significant difference was found between the two insemination techniques. When deep intra-uterine insemination was performed, semen was deposited with a rigid insemination device in the uterine horn, but very few experiments were performed with a flexible insemination device with which semen was deposited near the utero-tubal junction (Hawk et al., 1988). Theoretically, the insemination dose as used for conventional insemination in cattle (15 x 10^6 spermatozoa) could be reduced by at least 100-fold if semen was deposited near the UTJ (Hunter, 2001). This would mainly be due to the decreased loss of spermatozoa (Hunter, 2001). In the nineties, the possibility to sort semen of farm animals in X- and Y-bearing spermatozoa led to new interests in deep insemination (Johnson, 1991; Cran et al., 1993). In pigs and horses, pregnancy cannot be achieved with low doses of non-sexed semen (5 x 10^6) or with sex-sorted semen by means of conventional insemination, but pregnancy can be established with UTJ insemination
In horses, a distinctive advantage of semen deposition directly on the ostium uterinum tubae (utero-tubal papilla) rather than in the uterine body has been shown when the number of spermatozoa was reduced to 3 million (Morris et al., 2000). To be sure that the semen is deposited on the utero-tubal junction, the insemination is performed by means of a hysteroscope (Lindsey et al., 2002). To enable deposition of semen near the utero-tubal junction (UTJ) in sows, specially designed flexible insemination devices have been developed (Martinez et al., 2001; Grossfeld et al., 2003). Due to the curvature of the uterine horns in these species at the moment of insemination, no rigid insemination device can be used for UTJ insemination. In studies by Rath et al. (2003b) and Grossfeld et al. (2003), sows recently produced piglets after UTJ insemination with $5 \times 10^6$ sex-sorted spermatozoa. This is 2.5% of the conventional insemination dose of non-sexed semen. In bovines, as in horses and pigs, deep insemination is advised in cases of low dose insemination (< 5 million frozen-thawed spermatozoa) or with low fertile semen. However, up to now, no beneficial effect of deep insemination could be established (Seidel et al., 1999). The fact that only heifers were used in these studies may largely explain the absence of any difference. Since the uterine size is bigger in older cows than in heifers, it is more likely that a beneficial effect of UTJ insemination will be observed in older cows.

A remarkable anatomical difference between farm animals and humans is the relatively bigger uterine body (cavity) and absence of uterine horns in humans. In humans, inseminations can be performed intravaginally, intracervically, pericervically with a cap, intra-uterine, intra-tubally or directly intra-peritoneally. However intra-uterine insemination (IUI) seems to be the method of choice in most studies (Oei et al., 1992; Ripps et al., 1994; Ombelet et al., 1995; Guzick et al., 1999). IUI is a simple, cheap and lowly invasive technique by which the inseminate is slowly injected, high up in the uterine cavity (Peterson et al., 1994). Deposition of semen near the UTJ is not performed in humans, however clinical trials have been performed in which sperm was directly deposited into the oviduct (fallopian sperm perfusion, FSP). FSP could be beneficial for treatment of patients with unexplained infertility, but in other cases of infertility FSP appeared to be equal to or perhaps inferior to IUI (Karande et al., 1995; Nuojua-Huttunen et al., 1997; Trout and Kemmann, 1999).
In contrast to farm animals, the UTJ in humans is shaped rather like a funnel and is not guarded by mucosal folds (Hafez and Black, 1969; Beck and Boots, 1974). So, while in animals the UTJ may act as a barrier, in humans spermatozoa may be guided right into the UTJ. Moreover, in humans there is no conclusive evidence for a distinct oviductal sperm reservoir such as that of farm animals (Williams et al., 1993). Although some human spermatozoa have been observed to stick to epithelium under certain conditions in vitro (Pacey et al., 1995), spermatozoa rarely have been observed to bind tightly to oviductal epithelium in vitro (Yeung et al., 1994; Murray and Smith, 1997).

CONCLUSION

IUI is a simple and successful technique for assisted reproduction in animals and humans. While IUI in human is performed for reasons of subfertility, mainly caused by poor sperm quality, IUI in farm animals was originally performed for sanitary reasons but later especially for economic reasons. However, since the introduction of sex-sorted semen in farm animals, the same problem of subfertility as in human has arisen. To optimize fertilization rates with low insemination doses and semen of poor quality, deposition of the semen near the UTJ has been proven to be successful in sows and mares. As in sows and mares, UTJ insemination in cattle could be advantageous for insemination with low doses of semen and with semen of poor quality.
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CHAPTER 3

COLLECTION AND COMPOSITION OF CAUDA EPIDIDYMAL PLASMA IN THE BULL
ABSTRACT

When artificial insemination (AI) is performed in cattle, much lower insemination doses are required with fresh semen than with frozen-thawed semen to obtain similar pregnancy rates. However, a particular disadvantage of fresh semen is its limited shelf life. Since bovine spermatozoa can be stored for several weeks in the cauda epididymidis without negative effects on their fertilizing capacity, the cauda epididymidis is an interesting object to serve as a model in order to prolong the shelf life of fresh semen. In this study, bovine cauda epididymal fluid (CEF) was collected in vitro and in vivo, and the biochemical composition of the cauda epididymal plasma (CEP) was determined. Cauda epididymal fluid was collected in vitro by micropuncture of epididymides of slaughtered bulls. To evaluate the cannulation technique for the in vivo collection of CEF, cannulations were first performed in rams and the technique was subsequently applied in bulls. After centrifugation of the CEF, the CEP was separated and the biochemical composition of several ions and metabolites in the CEP was determined in a clinical laboratory. Finally, a new diluent with the same ionic composition, pH and osmolarity as the bovine CEP was developed and compared with the standard Tris diluent for extended preservation of fresh ejaculated semen. Storage of fresh ejaculated bovine semen in a diluent with the same ionic composition as the cauda epididymal plasma does not bring the ejaculated spermatozoa in a quiescent state nor extend their shelf life.
INTRODUCTION

The widespread use of artificial insemination (AI) in cattle can partially be attributed to the availability of suitable sperm diluents (Almquist, 1962; Shannon, 1964; Foote, 1970) and to the fact that small amounts of frozen-thawed spermatozoa (15 millions) can be deposited beyond the cervical barrier, in the uterus of the cow (Salisbury and Van Demark, 1961). Although great efforts have been made to optimize the freezing and thawing procedure of bovine spermatozoa, the cryopreservation process still has detrimental effects on semen quality (Watson, 1995; Woelders, 1997; Van Wagendonk-de Leeuw et al., 2000, Frijters and Luimstra-Mulder, 2003). Vishwanath et al. (1996) showed that in cattle 10 times more frozen-thawed spermatozoa were required to obtain similar pregnancy rates as with fresh semen. The ability to obtain good pregnancy rates with low insemination doses would increase the world wide spreading of genetically superior bulls. This is not only beneficial for the genetic progress in cattle breeding, but also increases the profits of the AI centres. However, one of the most important disadvantages of fresh semen is its limited storage capacity. Currently, fresh semen can be used for AI up to 3 days after collection when it is stored at room temperature, under N₂ gas in Caprogen® diluent (Livestock improvement corporation, Hamilton, New Zealand) (Vishwanath and Shannon, 1997). Since this storage period is rather short, more extended semen preservation time would decrease the work of sperm collection and would facilitate the distribution of fresh semen.

Based on the peculiar capacity of the cauda epididymidis to store bovine epididymal semen for several weeks without detrimental effects on the fertilizing capacity (Kirillov and Mozorov, 1936; Cascieri et al., 1976), we wanted to develop a new diluent for fresh ejaculated bovine semen based on the cauda epididymal conditions. The cauda epididymidis is characterized by low pH (Carr and Acott, 1984; Acott and Carr, 1984), hyperosmotic pressure of the fluid (Liu and Foote, 1998), high concentration of spermatozoa (Cascieri et al., 1976; Amann et al., 1976) low oxygen tension (Free et al., 1979), and low Na / K ratio (Crabo, 1965). Recently, genes responsible for the production of specific proteins present in CEP, maturation of epididymal spermatozoa, and secretion and absorption processes in the epididymis have been thoroughly investigated (Jones et al., 1998). However, only a few studies have been published concerning the ionic composition of cauda epididymal plasma (CEP) in bulls (Crabo, 1965; Amann et al., 1963).
Therefore, in this study, we first investigated the biochemical and more specifically, the ionic composition of the CEP collected by two different techniques. In vitro collection of CEP was performed by micropuncture of epididymides of slaughtered bulls. In vivo collection by means of cannulation was first performed on rams, and subsequently used in bulls. The effectiveness of the newly developed diluent based on the ionic composition of CEP was compared with the standard Tris diluent in a 6-days storage experiment.

MATERIAL AND METHODS

**Exp 1: In vitro collection of epididymal plasma**

Testicles of Belgian White-Blue beef bulls (aged 2-2.5) years were collected at the slaughterhouse, stored in an insulated container, and transported to the laboratory within 2 hours. Four collections of 100 epididymides per collection were performed. After removing superficial blood and interstitial fluid contamination, the pressure in the epididymal ducts was increased by clamping pincers on the proximal and distal part of the cauda epididymidis. Incisions were made in the connective tissue and care was taken to avoid small blood vessels. The epididymal fluid oozed out and was aspirated into a fine glass pipette and transferred into a small vial (Figure 1). To separate the sperm fraction from the plasma fraction, the epididymal fluid was centrifuged for 5 min at 3.214 x g. Subsequently, the superficial uncontaminated epididymal plasma fraction was removed and frozen at –20°C. Since the volume of CEP per testicle was much too low for individual analysis, cauda epididymal plasma of 100 epididymides was pooled.

Figure 1: Aspiration of epididymal plasma with a fine glass pipette from the cauda epididymidis of a bovine testicle.
**Exp 2: In vivo collection of epididymal plasma of ram and bull**

The institutional ethical committee for animal welfare of Ghent University approved all procedures involving the animals used in this study. The ram was used as a model for the bull to test the cannulation technique as previously reported by Amann et al. (1963) and Henault et al. (1995). Six Suffolk rams aged 1.5 to 2.5 years were used in this experiment, which was performed during the mating season (September-October). One week before cannulation, the quality of the ejaculated semen collected by means of an artificial vagina was assessed microscopically. For each ram, the progressive motility, the percentage of membrane intact spermatozoa, and the percentage of spermatozoa with normal morphology were at least 80%. Before the operation, rams were starved for 24 hrs but had access to water ad libitum. An intra-muscular injection of Xylazine (Xyl-M 2%, VMD, Arendonk, België) (1 ml/100 kg) was used as tranquillizer and combined with 15 ml of 2% procaine (VMD, Arendonk, België) as local anaesthesia. When the ram was tranquillized, he was fixed on the operation table. Subsequently, the scrotum was shaved, cleaned, and disinfected, and a sterile plastic cloth was used to keep the operation field clean.

A system of three cannulae which were axially movable into each other was used (Figure 2). This set-up was used to dilate the lumen of the ductus deferens without causing damage and to prevent cracking of the cannulae. To enable daily replacement of the collection vial, the cannulation set was equipped with a screw cap at one end.

![Figure 2: Different parts of the cannulation set: 1) inner tube, 2) outer tube, 3) protecting tube, 4) screw cap, 5) collection vial.](image)

To insert the cannulation system into the ductus deferens, a 6-8 cm incision was made in the caudal part of the scrotum immediately lateral to the septum scroti. The ductus deferens was exposed over a distance of 5 cm after making a small incision in the tunica vaginalis, and
isolated from the adjacent blood vessels and nerves by blunt dissection. A cross section was made in the ductus deferens and the inner silicone cannula was inserted into the lumen as far as possible (± 5 cm). An outer silicone cannula was placed completely over the inner cannula, fixed with glue and sutured to the ductus deferens and the tunica vaginalis by 4-0 silk sutures (Ethicon®, Neuilly, France). Removal of the inner cannula increased the free space in the outer cannula, and decreased the risk of obstruction. A protecting cannula (Tygon®, R3603, Fisher Bioblock Scientific, Tournay, Belgium) with a screw cap was placed over the outer cannula to prevent cracking. The cauda epididymal effluent was harvested via the indwelling outer cannula emptying into a sterile collection vial. The collection vial was replaced every 24h and after sexual stimulation of the animal. When signs of infection or discoloration of the epididymal contents were observed, the cannulation set was removed. The epididymal effluent was centrifuged for 5 min at 3.214 x g. The uncontaminated epididymal plasma was removed and frozen at –20°C. The epididymal plasma collected during the whole cannulation period, was pooled per individual animal.

For the cannulation of the bovine ductus deferens, 9 Holstein bulls aged 1.5 to 2.5 years were used. As in rams, one week before cannulation, the quality of the ejaculated semen was assessed and was good for all bulls. The same threshold levels were used as in the ram. The operation technique applied in the bull was identical to the technique described in the ram. In contrast to rams, bulls were cannulated at the opposite side when insufficient epididymal plasma was collected at the first cannulation side.

**Biochemical analysis of cauda epididymal plasma**

After collection and storage at –20°C, the biochemical composition of CEP of the ram and bull was analyzed. Cobas® Integra cassettes (Roche, Mannheim, Germany) were used for the analysis of Na, K, Cl, Ca, Mg, inorganic phosphate, bicarbonate, glucose, lactate and cholesterol. Osmolarity was evaluated with an osmometer (Fisk Associates®, Massachusetts, USA), and for the analysis of the pH, pH electrodes (pH 526, TUV®, Wilheim, Germany) were used.

**Storage of fresh bovine semen in Cauda Epididymal Plasma (CEP) diluent**

Fresh semen of 2 bulls with a normal sperm quality (progressive motility, percentage membrane intact spermatozoa, and percentage spermatozoa with normal morphology ≥ 80%) was collected by means of an artificial vagina in a falcon tube (Becton and Dickinson®, Le
Pont de Claix, France) containing 5 ml of Triladyl diluent, according to the method of De Pauw et al. (2003). Immediately after collection, the semen quality was evaluated, and subsequently the semen was centrifuged at 700 x g for 10 min. After centrifugation, the seminal plasma and Triladyl diluent were removed. Subsequently the fresh semen was stored in a liquid state at 5°C in the Tris and in a newly developed diluent based on the composition of the CEP. Both these media contained 10% egg yolk. The sperm was diluted to a concentration of $10 \times 10^6$ spermatozoa / ml and was stored in 1.8 ml cryovials (Nunc®, Wiesbaden, Germany) for 6 days. The sperm concentration was determined using a Bürker counting chamber by means of light microscopy (x 400).

The quality of the spermatozoa stored in Tris and in CEP diluent was evaluated before (t0), and after 2 (t2), 4 (t4), and 6 (t6) days of incubation. Membrane integrity was assessed by means of eosin-nigrosin staining and light microscopy (x 400). Total motility (TM) and progressive motility (PM) were assessed subjectively by 2 persons by means of light microscopy (x 200, 37°C). The Functional Sperm Concentration (FSC), Motile Sperm Concentration (MSC) and Sperm Motility Index (SMI) were determined in three replicates with the Sperm Quality Analyzer (SQA II-C®) (Orange Medical, Tilburg, The Netherlands). The SQA II-C® is provided of a photometric cell that registers the fluctuations in optical density and converts this information, through mathematical algorithms, digitally to a numerical output. The FSC represents the cells that are both motile and morphologically normal ($x 10^6$/ml), while the MSC represents the cells that are normally motile (concentration x % motility) in millions/ml. The SMI is expressing the overall sperm quality, taking into account three parameters, namely the concentration, the progressive motility, and the percentage spermatozoa with a normal morphology.

STATISTICAL ANALYSIS

The storage experiment was repeated 3 times. Data were analysed using a linear mixed effect model, with bull and repetition as random variables, day as group variable and allowing different standard deviations per stratum (S-plus 2000, Math Soft Inc, Cambridge, USA). Since residuals were not normally distributed for the FSC and MSC results, a log transformation was performed. Differences in sperm quality were considered as being significant when p-values were lower than 0.05.
RESULTS

**Exp 1: Collection of epididymal plasma by means of micropuncture from epididymides derived from the slaughterhouse**

When micropuncture was used, the cauda epididymal fluid of 100 epididymides could be collected by 2 persons within 2 hrs. However, only small amounts (115 ± 50 µL) of epididymal fluid were harvested per epididymis. The use of clamping pincers increased the pressure in the epididymal ducts, and facilitated the collection of the epididymal fluid. The cauda epididymal duct was clearly demarcated and micropuncture could be performed precisely. After centrifugation of the cauda epididymal fluid, only one fourth of the total volume was clear plasma which was aspirated and stored at –20°C until analysis.

**Exp 2: In vivo collection of epididymal plasma by means of cannulation of the ductus deferens**

In the ram, the cannulation technique of the ductus deferens was easy to perform. The ductus deferens could be located easily by palpation through the tunica vaginalis. Moreover, the consistency and straight pathway of the ductus deferens facilitated the insertion of the cannulation set. In the bull however, the ductus deferens was much more curled and insertion of the cannulation set up to the cauda epididymidis was more complicated. No epididymal fluid could be collected in one ram and two bulls which was probably due to trauma or inflammation caused by the operation. In two other bulls epididymal fluid was collected, but the amount of plasma obtained after centrifugation was not sufficient for biochemical analysis. Uncontaminated epididymal fluid could be obtained in rams for an average period of 17.2 ± 4.7 days, whereas this period was reduced to 9.2 ± 4.8 days in the bull. Variation in the daily collection volume occurred both in rams and bulls. In one bull, no epididymal fluid could be collected for 2 days due to obstruction of the cannula. Insertion of a small catheter and sexual stimulation resolved this problem. The average daily production of epididymal fluid after successful cannulation was 338.4 ± 113.6 µL and 426.3 ± 137.3 µL in rams and bulls, respectively. The total output of epididymal fluid during the entire collection period was 3941.0 ± 1020.2 µL and 4108.3 ± 2420.4 µL in the ram and in the bull, respectively.
**Analysis of the ionic composition of epididymal plasma**

The composition of the new CEP diluent was based on the ionic composition, pH, osmolarity and Na/K ratio of the bovine CEP as analysed in experiment 1 and 2 (Table 1).

Table 1: Composition of the cauda epididymal plasma in the ram and the bull after in vivo cannulation and after micropuncture of epididymides collected in the slaughterhouse.

<table>
<thead>
<tr>
<th></th>
<th>Units</th>
<th>Ram: cannulation</th>
<th>Bull: cannulation</th>
<th>Bull: micropuncture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>mmol/l</td>
<td>65.8 ± 15.9</td>
<td>42.3 ± 4.0</td>
<td>50.5 ± 6.7</td>
</tr>
<tr>
<td>K⁺</td>
<td>mmol/l</td>
<td>28.4 ± 4.4</td>
<td>28.9 ± 0.9</td>
<td>33.5 ± 4.1</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>mmol/l</td>
<td>44.5 ± 12.2</td>
<td>23.0 ± 1.9</td>
<td>38.6 ± 8.1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>mmol/l</td>
<td>0.65 ± 0.1</td>
<td>0.43 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>mmol/l</td>
<td>2.7 ± 0.6</td>
<td>2.0 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>P⁻</td>
<td>mmol/l</td>
<td>17.3 ± 3.4</td>
<td>14.7 ± 7.3</td>
<td>7.1 ± 1.5</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>mmol/l</td>
<td>2.6 ± 0.6</td>
<td>2.0 ± 1.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/l</td>
<td>0.08 ± 0.06</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mmol/l</td>
<td>1.7 ± 0.3</td>
<td>1.56 ± 0.35</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Lactate</td>
<td>mmol/l</td>
<td>1.4 ± 0.8</td>
<td>1.50 ± 1.2</td>
<td>12.1 ± 3.6</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6.7 ± 0.3</td>
<td>6.7 ± 0.3</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>osm</td>
<td>mOsm</td>
<td>347.5 ± 16.4</td>
<td>338.5 ± 23.0</td>
<td>338.7 ± 12.2</td>
</tr>
<tr>
<td>Na/K ratio</td>
<td></td>
<td>2.3</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

ND = not determined

The ions were administered to the CEP diluent as salt solutions. The concentration of each ion (Na⁺, K⁺, Cl⁻, Ca²⁺, P⁻ and Mg²⁺) in the CEP diluent ranged between the concentrations found in the bovine CEP after cannulation and micropuncture (Table 2).
Table 2: Composition of the CEP diluent based on the ionic composition of the cauda epididymal plasma (CEP) of the bull.

<table>
<thead>
<tr>
<th></th>
<th>Units</th>
<th>CEP diluent</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>mmol/l</td>
<td>44.9</td>
<td>42.3 ± 4.0</td>
</tr>
<tr>
<td>K⁺</td>
<td>mmol/l</td>
<td>29.0</td>
<td>28.9 ± 0.9</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>mmol/l</td>
<td>36.8</td>
<td>23.0 ± 1.9</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>mmol/l</td>
<td>0.8</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>mmol/l</td>
<td>1.6</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>P⁻</td>
<td>mmol/l</td>
<td>8.0</td>
<td>14.7 ± 7.3</td>
</tr>
<tr>
<td>Na⁺/K⁺ ratio</td>
<td></td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6.6</td>
<td>6.7</td>
</tr>
<tr>
<td>Osm</td>
<td>mOsm</td>
<td>325.0</td>
<td>338.5</td>
</tr>
</tbody>
</table>

To increase the osmolarity of the CEP diluent without affecting the ion concentrations, sorbitol was administered to the CEP diluent. Since the CEP diluent was developed for extended semen preservation, extra fructose and Tris were administered. The supplementation of extra fructose and Tris had to prevent that the energy supply or buffering capacity of the medium would be a limiting factor for the survival of the spermatozoa. In table 3, the exact biochemical composition of the Tris and CEP diluent is represented. In this study, the Tris diluent is used as reference diluent since it is the most common diluent for fresh bovine semen in Europe. To obtain the right ionic composition, sodium nitrate and potassium nitrate were added. In general, the salt composition of the CEP diluent was more complex than in the Tris diluent.
Table 3: Biochemical composition of the Tris and CEP diluent used in the storage experiment.

<table>
<thead>
<tr>
<th></th>
<th>Units</th>
<th>Tris diluent</th>
<th>CEP diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl</td>
<td>mmol/l</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>KCl</td>
<td>mmol/l</td>
<td>0</td>
<td>22.0</td>
</tr>
<tr>
<td>MgCl</td>
<td>mmol/l</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>NaCl</td>
<td>mmol/l</td>
<td>11.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Na-nitrate</td>
<td>mmol/l</td>
<td>0</td>
<td>18.0</td>
</tr>
<tr>
<td>K-nitrate</td>
<td>mmol/l</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>Na-bicarbonate</td>
<td>mmol/l</td>
<td>11.9</td>
<td>11.9</td>
</tr>
<tr>
<td>Na-phosphate</td>
<td>mmol/l</td>
<td>0</td>
<td>5.0</td>
</tr>
<tr>
<td>K-phosphate</td>
<td>mmol/l</td>
<td>0</td>
<td>3.0</td>
</tr>
<tr>
<td>glucose</td>
<td>mmol/l</td>
<td>11.1</td>
<td>0</td>
</tr>
<tr>
<td>fructose</td>
<td>mmol/l</td>
<td>55.0</td>
<td>55.0</td>
</tr>
<tr>
<td>sorbitol</td>
<td>mmol/l</td>
<td>0</td>
<td>5.5</td>
</tr>
<tr>
<td>BSA</td>
<td>g/l</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Tris</td>
<td>mmol/l</td>
<td>133.7</td>
<td>133.7</td>
</tr>
<tr>
<td>genta-S</td>
<td>g/l</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>egg yolk</td>
<td>%</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>citric acid</td>
<td>mmol/l</td>
<td>42.9</td>
<td>42.9</td>
</tr>
<tr>
<td>glycine</td>
<td>mmol/l</td>
<td>83.4</td>
<td>0</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>7.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Osm</td>
<td>mOsm</td>
<td>325</td>
<td>325</td>
</tr>
</tbody>
</table>

**Storage of fresh bovine semen in Cauda Epididymal Plasma (CEP) diluent**

At the start of the incubation (t0), all spermatozoa expressed a good progressive and total motility (85%) in both the Tris and CEP diluent. However, a significant decrease over time of total and progressive motility, and membrane integrity was observed both media (p < 0.05). No difference in membrane integrity (p = 0.25) and progressive motility (p = 0.57) was found between spermatozoa stored in Tris and CEP diluent. The total motility decreased more rapidly for spermatozoa stored in the CEP diluent than in the Tris diluent (p = 0.01).

The first 2 days of incubation, no difference was observed in total motility between spermatozoa stored in both diluents, while the progressive motility was slightly better for spermatozoa stored in the CEP diluent. These findings were confirmed by the slightly higher sperm motility values (SMI, FSC and MSC) obtained with the SQA for spermatozoa stored in the CEP diluent than in the Tris diluent (Figure 3).
From four days of incubation onwards, spermatozoa stored in the the Tris diluent had higher values for total motility, SMI, FSC, and MSC than spermatozoa stored in the CEP diluent. While the total motility of spermatozoa stored in the Tris and CEP diluent at t6 remained rather high (65% and 48% respectively), the progressive motility decreased to 25% and 13% respectively. In the Tris diluent, higher sperm motility parameters were obtained with the SQA-II C® at t4 and t6 than at t0 and t2. This is in contrast to the total and progressive motility, which decreased significantly over time in the Tris diluent.

DISCUSSION

In this study, the biochemical composition of the CEP of the bull was evaluated using both in vitro (micropuncture) and in vivo (cannulation) collection techniques. Rams were used as model for the in vivo cannulation of the ductus deferens in the bull. Each of the two collection methods had both advantages and disadvantages.

Using the micropuncture method, large numbers of epididymides can be processed within a short period of time. However, contamination of the epididymal plasma with interstitial fluid, blood and epithelial cells may occur. The degree of cellular contamination can be minimized by means of centrifugation, while contamination with interstitial fluid can be reduced to less than 5% when the collection method is carried out carefully (Crabo, 1965). In the present study, the contamination with interstitial fluid was minimized by careful dissection of the cauda epididymidis, a good clean-up of the surface and an accurate micropuncture of the cauda epididymal duct using a very fine pipette. Besides contamination, other disadvantages of the micropuncture method are: 1) the small quantities of CEP per epididymis, and 2) the fact that samples can be harvested only once. Regarding the small amount of epididymidal plasma available per epididymis, no extensive individual biochemical
Collection and composition of cauda epididymal plasma in the bull

analysis per bull can be performed. Consequently, when large amounts of epididymal plasma or spermatozoa of an individual are required, or when a follow up of the characteristics of epididymal plasma or spermatozoa over time has to be performed, micropuncture is not a useful collection method (Amann et al., 1963). In these cases, cannulation of the ductus deferens is a better alternative.

Cannulation of the ductus deferens in the bull was first described by Amann et al. (1963). Anatomical differences make the cannulation of the ductus deferens easier to perform in the ram than in the bull, which was also the case in the present study since more complications were encountered in the bull than in the ram. In two bulls no epididymal fluid could be collected, while in two other bulls epididymal plasma could be collected, but insufficient to allow biochemical analysis. Even in the bulls which had been cannulated successfully, the patency periods of the cannula were shorter than in the ram (9.2 ± 4.8d vs 17.2 ± 4.7d). However, the daily and total epididymal fluid productions were higher in the bull than in the ram, which is possible due to the larger size of the bovine testicles. Another advantage is the fact that during unilateral cannulation of the ductus deferens, ejaculated semen can be collected simultaneously. This enables the comparison of epididymal sperm and plasma characteristics with these of ejaculated sperm and plasma, and the effect of addition of accessory sex gland fluid on epididymal spermatozoa (Henault et al., 1995).

The number of studies in which the ionic composition of the cauda epididymal plasma in the bull is determined is very limited (Crabo, 1965; Wales et al., 1966; Mann, 1964). In the ram, the composition of the CEP was first described by Scott et al. (1963). Compared to our study (Table 2), they found lower concentrations for the different ions, for the Na/K ratio, and for the urea and total protein concentration. However, the lactate concentrations reported by Scott et al. (1963) were 5 times higher than in our study. The differences in ion concentration may be the result of the difference in collection method and analysis technique used to determine the biochemical composition of the CEP. In the study of Scott et al. (1963) the surface of the cauda epididymal duct was scraped in order to collect epididymal fluid, resulting in contamination with interstitial fluid. The interstitial fluid might have a dilution effect and explain the lower concentration of the different ions in their study. The increased K concentration in the CEP reported by Scott et al. (1963) can be due to the cell lesions caused by scraping the surface to collect CEP. The increased lactate concentrations may be due to the metabolism of glucose present in the CEP. The difference in urea and total protein concentration might be related with the difference in diet.
In our study, the biochemical composition of the CEP in the bull was examined with the aim to develop a suitable diluent for prolonged preservation of ejaculated semen. The CEP in our study as well as in that of Crabo (1965) and Wales et al. (1966) was collected by means of micropuncture, but nonetheless clear differences in ion concentrations were observed between our study and the previous ones (Verberckmoes et al., 2001). A great deal of the variation may be due to the method of biochemical analysis and to the fact that different breeds of bulls were used.

The composition of the new diluent (CEP diluent) was based on the pH, osmolarity, concentrations of Na, K, Cl, Ca, Mg and inorganic P, and on the Na/K ratio in the CEP as it occurs in the bull (Table 2).

The pH of the CEP after cannulation and micropuncture was 6.4 and 6.7 respectively, and is about the same as in the stallion, the boar, the ram, the dog, and the rabbit (Jones, 1978). Carr and Acott (1984) however, reported that the pH of the CEP of the bull is as low as 5.8 and in combination with lactate could be responsible for the reversible inhibition of the motility of the spermatozoa in the cauda epididymidis. This is in contrast with storage experiments performed earlier in our laboratory with ejaculated semen (De Pauw et al., 2003). When ejaculated semen was stored in Hepes-TALP at a pH lower than 6, the motility of the spermatozoa dropped to zero within 24 hrs, and no membrane intact spermatozoa could be detected after 72 hrs of incubation. When the pH of the storage medium increased from 6 to 8, no significant difference in sperm motility was observed, while the membrane integrity decreased significantly (De Pauw et al., 2003). In the present study, no difference in membrane integrity was observed between spermatozoa stored in Tris (pH = 7.5) vs. CEP diluent (pH = 6.6), while the motility decreased more rapidly in the CEP diluent.

The slight hypertonicity of the CEP as determined in this study (338 mOsm) is in agreement with previous findings (Liu and Foote, 1998; Scott et al., 1963; Cragle and Salisbury, 1959; Levine and Marsh, 1971). Especially in hibernating bats, where a hypertonicity of 1500 mOsm is reached, this is assumed to be the most important parameter that enables prolonged preservation of semen as a result of dehydration (Crichton et al., 1994). The possibility to increase survival of ejaculated bull sperm in hypertonic solutions is however very limited (De Pauw et al., 2003).

Besides low pH and hypertonicity of the medium, different inorganic and organic constituents have been alleged to be responsible for maintaining sperm in quiescence (Verma,
We have tried to mimic the inorganic components (Na, K, Cl, Ca, and Mg) of the CEP in our diluent. Among the inorganic constituents, the effect of different ions as present in the cauda epididymidis upon keeping epididymal spermatozoa in a quiescent state has been investigated in several species. Morton et al. (1978) showed that low levels or absence of calcium was beneficial for sperm quiescence, while Crabo (1965) reported that especially a low Na/K ratio was required to keep the spermatozoa in a quiescent state. According to Cragle and Salisbury (1959), a high K/Ca ratio was required besides a low Na/K ratio to inhibit spermatozoal respiration and glycolysis. However, despite the fact that the ion concentrations, the Na/K ratio and the K/Ca ratio of our CEP diluent were identical to those of the CEP, no reduction of the spermatozoal activity was observed (Figure 3). Moreover, during the first 2 days of storage, even higher sperm motility values were obtained for spermatozoa stored in the CEP diluent than for spermatozoa stored in the Tris diluent. After 2 days, the Tris diluent was superior to the CEP diluent for sperm survival in vitro.

Sorbitol, which is also present in seminal plasma, was added to the CEP diluent for its osmotic effect and can serve as a limited extent as a natural substrate for semen under aerobic condition (Crichton et al., 1994; Clegg et al., 1986). Other organic components of epididymal fluid, such as glycerylphosphorylcholine and carnitine were not added to our CEP diluent. Their effect has been studied in rats, with good result on sperm quiescence (Turner and Giles, 1982). However, when carnitine was added to medium conditioned by epididymal cells and used for bull semen preservation, no beneficial effect on sperm survival could be observed (Reyes-Moreno et al., 2000).

Since sperm fertility declines much faster than sperm motility (Vishwanath and Shannon, 1997, 2000), and given the fact that after 2 days of incubation sperm motility was lower for spermatozoa stored in the CEP than in the Tris diluent, it can be concluded that the CEP diluent is not appropriate for extended preservation of fresh bovine semen. Moreover, despite the fact that the CEP diluent had the same ionic composition, osmolarity, and pH as the CEP in the bull, no quiescent state or extended life span of the ejaculated spermatozoa was acquired. Future experiments to establish a superior sperm diluent for fresh bovine semen are directed towards the exclusion of nitrate-salts with concomitant modification in ionic composition of the CEP diluent and to modifications in egg yolk and polyol content, and the temperature of storage.
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Levine N, Marsh DJ. Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in the rat. J Physiol 1971; 213: 557-570.


CHAPTER 4

STORAGE OF FRESH BOVINE SEMEN IN A DILUENT BASED ON THE IONIC COMPOSITION OF CAUDA EPIDIDYMAL PLASMA (CEP)

Verberckmoes S., Van Soom A., Dewulf J., De Pauw I., de Kruif A.
ABSTRACT

For artificial insemination (AI) in cattle, much lower insemination doses can be applied when fresh semen is used instead of frozen-thawed semen. However, a particular disadvantage of fresh semen is its limited shelf life. Since bovine spermatozoa can be stored for several weeks in the cauda epididymidis without negative effects on their fertilizing capacity, the cauda epididymidis can serve as a model in order to prolong the shelf life of fresh semen. First, the ionic composition of the CEP-1 diluent which had the same ionic composition, pH and osmolarity as the bovine cauda epididymal plasma was modified (CEP-2) and the storage capacity of the CEP-2 diluent was compared with this of the CEP-1 and Tris diluent. Subsequently, the effect of addition of different polyols (sorbitol, glycerol, mannitol) and egg yolk concentrations (5, 10 and 20%) to the CEP 2 diluent was assessed.

Sperm quality decreased rapidly in the CEP-1 diluent. The quality and especially progressive motility of spermatozoa stored in the CEP-2 diluent were better than in the CEP-1 and Tris diluent. No significant effects of different sugars or egg yolk concentrations on the quality of fresh bovine semen in the CEP-2 diluent were observed. In conclusion, the CEP-2 diluent with 10% egg yolk and 1g/L sorbitol may be used for extended preservation of fresh bovine semen at 5°C up to 6 days.
INTRODUCTION

The widespread use of artificial insemination (AI) in cattle can partly be attributed to the availability of suitable diluents (Almquist, 1962; Shannon, 1964; Foote, 1970) and to the fact that similar pregnancy rates can be obtained with small amounts of frozen-thawed semen (± 15 millions) by means of AI as by means of natural mating without the risk of spreading venereal diseases (Salisbury and Van Demark, 1961). In contrast to natural mating, the cervical barrier is by-passed when AI is performed, and semen is deposited directly into the uterine body. In this way, AI in cattle enables a more than 100-fold reduction of the insemination dose compared to natural mating without a decrease in pregnancy rates (Hunter, 2003). The ability to obtain high pregnancy rates with low insemination doses increases the number of insemination doses produced by genetically superior sires and accelerates the genetically progress in livestock. Currently, more than 95% of semen in cattle is processed as frozen product (Chupin and Thibier, 1995), while less than 5% is processed as fresh semen. The use of fresh semen is predominantly restricted to New Zealand, and smaller amounts are used in Africa, France, Australia, Germany, and Eastern Europe (Thibier and Wagner, 2000). The use of frozen-thawed semen facilitates worldwide distribution and enables screening of the bull for infectious diseases before its semen is used in the field. However, in cattle the insemination dose for AI can be reduced from 15 to 2 to 4 millions of spermatozoa without a reduction in pregnancy rates when fresh instead of frozen-thawed spermatozoa are used (Vishwanath et al., 1996). The much higher insemination dose required with frozen-thawed than with fresh semen is the result of the detrimental effect of the freezing and thawing procedure on semen quality (Holt, 2000). When AI is performed with liquid bovine semen in Europe, semen is traditionally stored at 5°C in a Tris-glycerol based extender which is normally used for freezing semen (De Leeuw et al., 1993). In New Zealand however, most of the liquid semen is stored at room temperature, under N₂ gas in Caprogen® diluent (Vishwanath and Shannon, 1997). Semen stored in these diluents, is advised to be used within 3 days. Obviously, the short shelf life is the most important disadvantages of fresh semen. Therefore, extension of the shelf life of fresh semen would be interesting for the cattle breeding industry for several reasons; 1) more insemination doses could be produced per ejaculate, 2) the work load during sperm collection and processing would be decreased, and 3) the distribution of liquid semen would be simplified.
Chapter 4

Inspired by the specific capacity of the bovine cauda epididymidis to store spermatozoa for several weeks without reduction of their fertilizing capacity (Kirillov and Mozorov, 1936; Cascieri et al., 1976), we have developed a sperm diluent largely based on the ionic composition of bovine cauda epididymal fluid. Despite the fact that for in vitro culture of preimplantation embryos several synthetic culture media have been developed based on the composition of oviductal fluid, such as ovine synthetic oviduct fluid (SOF) (Tervit et al., 1972), human tubal fluid (Quinn et al., 1985) and pig zygote medium (Yoshioka et al., 2002), to our knowledge no such approach has ever been used in the development of a sperm diluent. To analyse the ionic composition of cauda epididymal fluid we have cannulated the cauda epididymidis of bulls in vivo and performed micropuncture of slaughterhouse epididymides (Verberckmoes et al., 2001). In this study we describe the composition of the Cauda Epididymal Plasma (CEP) diluent, including some of the adjustments which were necessary to optimize its sperm storage capacity and we compare sperm survival in CEP and Tris diluent. In addition, the effect of different concentrations of egg yolk and polyols on sperm survival in the CEP diluent are examined.

MATERIAL AND METHODS

Collection and preparation of semen

Fresh semen of 3 bulls with normal sperm quality (progressive motility, percentage of membrane intact spermatozoa, and percentage of spermatozoa with normal morphology ≥ 80%) was collected by means of an artificial vagina in a falcon tube (Becton and Dickinson®, Le Pont De Claix, France) containing 5 ml of Triladyl® diluent (Minitüb, Tiefenbach, Germany), according to the method described by De Pauw et al. (2003a). Immediately after collection, semen was centrifuged at 700 x g for 10 min. After centrifugation, the seminal plasma and Triladyl® diluent were removed. The sperm concentration was determined using a Bürker counting chamber and light microscopy (x 400). Spermatozoa were stored at a concentration of 10 x 10⁶ /ml in the different sperm diluents. Spermatozoa were incubated for 6 or 8 days in 1.8 ml cryovials (Nunc®, Wiesbaden, Germany) at 5°C in darkness.

Media

Chemicals were obtained from Sigma (Bornem, Belgium) and Life Technologies, Gibco BRL® products (Merelbeke, Belgium). Triladyl® diluent (Minitüb, Tiefenbach, Germany) was
used for the dilution and coating of the ejaculated spermatozoa (De Pauw et al., 2003a, b).

Newly developed cauda epididymal plasma (CEP) diluent was used as the basal sperm diluent (Table 1). The inorganic composition of the CEP-1 medium was calculated according to the ionic concentration of the cauda epididymal fluid as reported previously (Verberckmoes et al., 2001). Since preliminary experiments showed that the presence of nitrate in CEP-1 was detrimental for sperm survival, nitrates were omitted from CEP-2, and Ca, P, and Mg concentrations were raised. In both CEP-1 and CEP-2, no glucose but fructose and citric acid were added to the basal medium as energy substrates, at a concentration comparable to that in TRIS. Sorbitol was added to the CEP-1 and CEP-2 to increase the osmolarity to the same level as that in cauda epididymal plasma, while BSA was added as a macromolecular component. TRIS diluent was used for comparison.

Table 1: Composition of sperm diluents.

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Sperm quality assessment

The sperm quality parameters were evaluated at day 0 (t0), 2 (t2), 4 (t4), 6 (t6), and 8 (t8). Membrane integrity was determined in 100 spermatozoa by means of nigrosin eosin staining and light microscopy (x 400). Total motility (TM), progressive motility (PM), and hyperactivation of the spermatozoa were assessed subjectively on a heated stage (37°C) by 2 persons and by means of light microscopy (x 200). Spermatozoa were estimated as being hyperactivated when their motility was extremely vigorous and with high curvature flagellar movements. The Functional Sperm Concentration (FSC), Motile Sperm Concentration (MSC) and Sperm Motility Index (SMI) were determined objectively in three replicates with the Sperm Quality Analyzer (SQA II-C®) (Orange Medical, Tilburg, The Netherlands). The SQA II-C® is equipped with a photometric cell that registers the fluctuations in optical density and converts this information, through mathematical algorithms, digitally to a numerical output. The functional sperm concentration (FSC) expresses the number of spermatozoa that are both motile and morphological normal, while the motile sperm concentration (MSC) expresses the number of spermatozoa with a normal motility (concentration x motility percentage). The sperm motility index (SMI) presents the overall sperm quality, taking into account: the concentration, the progressive motility, and the percentage of spermatozoa with a normal morphology.

Experiment 1: Quality assessment of spermatozoa stored in the CEP-1 diluent, the CEP-2 diluent, and the Tris diluent

To compare sperm survival in both versions of the CEP diluent with that in the TRIS diluent, freshly ejaculated semen of 3 bulls was collected, centrifuged and subsequently stored in the 3 diluents for 6 days at 5°C. Sperm quality was assessed every two days as described above.

Experiment 2: Effect of sorbitol, glycerol, and mannitol on sperm survival in CEP-2 diluent.

For further experiments, only CEP-2 was used. To determine the effect of different polyols on the storage capacity of CEP-2 diluent, which contains 1 mg/ml of sorbitol, its storage capacity was compared with this of CEP-2 diluent without sorbitol, and with CEP-2 diluent in which sorbitol was replaced by the same concentrations of glycerol or mannitol. Freshly ejaculated semen of the 3 bulls was stored in the 4 diluents for 8 days and sperm quality was assessed every two days.
**Experiment 3: Quality assessment of spermatozoa stored in CEP-2 diluent with sorbitol and different egg yolk concentrations.**

After collection and centrifugation, freshly ejaculated bovine semen was diluted in CEP-2 diluent with 1 mg/ml of sorbitol and 3 different concentrations of egg yolk: 5%, 10%, and 20%. The diluted semen was stored for 8 days at 5°C, and sperm quality was assessed every 2 days as described before.

**STATISTICAL ANALYSIS**

All experiments were repeated 4 times. Data were analysed using a linear mixed effect model, with bull as random variable and allowing different standard deviations per stratum (S-plus 2000, Math Soft, Inc, Cambridge, USA). An autoregressive correlation structure of the first order was used to account for correlation in the data. Since residuals were not normally distributed for the FSC and MSC results, a log transformation was performed. Differences in sperm quality were considered as being significant when p-values were lower than 0.05.

**RESULTS**

**Experiment 1: Quality assessment of spermatozoa stored in the CEP-1 diluent, the CEP-2 diluent, and the Tris diluent.**

Membrane integrity, total and progressive motility, significantly decreased over time for spermatozoa stored in each of the 3 diluents (p < 0.01). However, due to the high variation in SMI, MSC, and FSC values, no decrease over time could be found for these parameters. Sperm membrane integrity did not differ between the 3 diluents, while significantly higher total motility values were obtained for spermatozoa stored in Tris diluent than for spermatozoa stored in the other diluents (p < 0.01) (Figure 1). Notwithstanding no significant difference in progressive motility was found between the 3 diluents (p = 0.57), progressive motility of spermatozoa stored in CEP-2 diluent tended to be higher than in both other diluents (Figure 1). When sperm motility was subjectively evaluated, a remarkably high percentage of hyperactivated spermatozoa (20 to 30%) was observed in Tris diluent, while no hyperactivated spermatozoa were observed in CEP-1 and CEP-2 diluent. Significant
differences (p < 0.01) in SMI, MSC, FSC for spermatozoa stored in the 3 different diluents were found, however there was no consistency in which of the 3 diluents was the best.

**Experiment 2: Effect of sorbitol, glycerol, and mannitol on sperm survival in CEP-2 diluent.**

For spermatozoa stored in CEP-2 diluent with different polyols, all evaluated sperm quality parameters decreased significantly over time (p < 0.01). A significant difference in total (p = 0.01) and progressive sperm motility (p < 0.01) was observed between the different media. The best results were obtained in CEP-2 diluent with sorbitol and glycerol (Figure 2). After 6 days of storage, about 40% of the spermatozoa stored in CEP-2 diluent containing sorbitol or glycerol were still progressively motile. In CEP-2 diluent without polyols however, less than 25% of the spermatozoa were progressively motile at day 6. For membrane integrity (p = 0.72), FSC (p = 0.14), MSC (p = 0.18), and SMI (p = 0.30) no differences were found between the different media. Since good results were obtained with CEP-2 diluent with sorbitol, this diluent was used in further experiments.

Figure 2: Percentage of progressive motility (Prog. Mot.) and sperm motility index (SMI) of fresh bovine semen stored for 8 days at 5°C in CEP-2 diluent without polyols, with sorbitol (1 mg/ml), glycerol (1 mg/ml), or mannitol (1 mg/ml).
Experiment 3: Quality assessment of spermatozoa stored in CEP-2 diluent with sorbitol and different egg yolk concentrations.

All evaluated sperm quality parameters decreased significantly over time in CEP-2 diluent with 5%, 10% and 20% egg yolk (p < 0.01). However, no significant differences in membrane integrity (p = 0.44), total (p = 0.68) and progressive motility (p = 0.56) were detected between CEP-2 diluents with different egg yolk concentrations (Figure 3). The percentage of membrane intact spermatozoa decreased from 91.4 ± 3.6 at t0 to 80.8 ± 9.0, 78.3 ± 10.1, and 75.8 ± 8.9 at t8 in CEP-2 diluent with 5%, 10% and 20% egg yolk respectively. In none of CEP-2 diluents, hyperactivated spermatozoa were observed and progressive motility was still 40% after 6 days of storage at 5°C (Figure 3). After 6 and 8 days of storage, highest FSC, MSC, and SMI values were obtained for spermatozoa stored in CEP diluent with 10% egg yolk (Figure 3).

Figure 3: Percentage of progressive motility (Prog. Mot.) and sperm motility index (SMI) of spermatozoa stored for 8 days at 5°C in CEP-2 diluent with sorbitol and with 5%, 10%, and 20% egg yolk.

DISCUSSION

Despite the fact that we were not able to get the ejaculated spermatozoa in a quiescent state, our results clearly indicate that our novel CEP diluent is efficient for long term preservation of bovine spermatozoa. For the first time, semen has been preserved in vitro in a diluent based on the composition of epididymal fluid. Bovine spermatozoa can be stored in the cauda epididymidis in a quiescent state for several weeks without reduction of their fertilizing capacity. The peculiar capacity of the cauda epididymidis to keep spermatozoa in a quiescent state has been attributed to different conditions present in the cauda epididymal plasma (CEP). In the studies by Carr and Acott (1984) and Acott and Carr (1984) inhibition of bovine spermatozoa was attributed to the interaction between pH and a quiescence factor. Other studies attributed the quiescent state of the epididymal spermatozoa to specific ion
concentrations and ion ratios in the CEP. Morton et al. (1978) found a correlation between the amount of free calcium in the cauda epididymal plasma of mammals and the level of sperm motility, while Crabo (1965) reported that in bovine especially the low Na/K ratio would be responsible for keeping spermatozoa in a quiescent state. According to Cragle and Salisbury (1959), the inhibition of spermatozoal respiration and glycolysis in the bovine epididymis would be obtained by the high K/Ca ratio in combination with the low Na/K ratio. However, despite the fact that the pH, the ion concentrations, the Na/K ratio and the K/Ca ratio of CEP-1 diluent were identical to those of the bovine cauda CEP (Verberckmoes et al., 2001), no reduction in spermatozoal activity could be observed, indicating that factors other than the ionic composition of the medium are essential.

Another characteristic of the cauda epididymidis is that spermatozoa are stored at a very high concentration (1 to 3 x 10⁹ spermatozoa/ml). However, in vitro experiments with ejaculated semen have shown that better results are obtained at a concentration of 10 x 10⁶ than at 100 or 500 x 10⁶ spermatozoa/ml (De Pauw et al., 2003).

Moreover, a combination of different factors might be responsible for the impracticability to bring the ejaculated spermatozoa back into a quiescent state. At the moment of ejaculation, spermatozoa previously stored in the cauda epididymidis are mixed with seminal plasma secreted by the accessory sex glands (seminal vesicles, prostate gland and Cowper’s gland) and become increasingly motile. Besides this increase in motility, ejaculated spermatozoa also differ from the epididymal spermatozoa in intracellular pH (Moore and Hibbitt, 1975; Hammerstedt et al., 1979), respiration (Lardy and Ghosh, 1952), ATP synthesis and consumption (Cascieri and Amann 1976), heparin-binding sites (Nass et al. 1990), and the profile of proteins bound to the plasma membrane (Lee et al., 1985; Shivaji, 1986; Calvete et al., 1996). All these factors together may be responsible for the irreversible activation of the quiescent epididymal spermatozoa (Morton et al., 1978; Cragle and Salisbury, 1959; Lardy and Ghosh, 1952). Notwithstanding the fact that spermatozoa were coated in a Triladyl® diluent (De Pauw et al., 2003a, b), that the seminal plasma together with the Triladyl® diluent was removed immediately after collection, and that spermatozoa were incubated in a medium with a biochemical composition comparable to this of the bovine cauda epididymal plasma, activation of the quiescent epididymal spermatozoa could not be prevented.

Since the quiescent state of the spermatozoa could clearly not be achieved, special attention was paid to design a diluent which could preserve progressive motility of
Storage of fresh bovine semen in CEP-1 diluent 73

spermatozoa as long as possible, since this it is one of the best indicators to predict in vivo fertility (Zhang et al., 1998; Correa et al., 1997; Verberckmoes et al., 2002a).

In CEP-2 diluent the Ca, Mg, and inorganic P concentrations were increased, compared to CEP-1 diluent. These changes had no effect on sperm membrane integrity and total motility, however the progressive motility was clearly improved. The beneficial effect of increased Ca, Mg, and P concentrations in CEP-2 diluent on the progressive motility may be due to the improved activity of the Ca and Mg dependent enzyme, adenylate cyclase, in the spermatozoa (Garbers et al., 1973; Garbers and Kopf, 1978; Lapointe et al., 1996). The higher activity of adenylate cyclase results in increased cAMP concentrations, which makes it possible to raise and prolong the progressive sperm motility significantly (Mann and Lutwak-Mann, 1981). A disadvantage of high Ca concentrations in the incubation medium can be that it may cause a rapid influx of calcium ions, and trigger the acrosome reaction which restricts the shelf life of the spermatozoa (Zhang et al., 1991; Christensen et al., 1996). Notwithstanding the Ca concentration in CEP-2 was higher than in CEP-1, the shelf life of the spermatozoa in CEP-2 was increased. This can be explained by the simultaneous increase of Mg, which inhibits the Ca induced acrosome reaction as shown in guinea pig and hamster spermatozoa (Talbot, 1975). Besides acrosome reaction, high concentrations of Ca can also induce hyperactivation. However, in this study no hyperactivated spermatozoa were observed in CEP-2 diluent which may be due to the fact that not only high Ca concentrations, but also sufficient ATP and an alkaline environment are required to induce hyperactivation (Ho et al., 2002). The higher total motility for spermatozoa stored in Tris diluent than in CEP-2 diluent, and opposite results for the progressive motility can be explained by the absence of hyperactivated spermatozoa in CEP-2 diluent.

When the effect of different polyols was compared, it was obvious that progressive sperm motility outcomes were highest for spermatozoa diluted in CEP-2 diluent with sorbitol or glycerol. Originally, sorbitol was added to CEP diluent in order to 1) increase the osmolarity of the medium to the same level as in the CEP of the bull (338 mOsm), and 2) as thermal protector, since the CEP diluent was developed for sperm storage at 5 °C. In previous studies performed in our laboratory, the effect of increasing osmolarity by addition of sorbitol on sperm quality was examined, and we could conclude that the exact osmolarity of the storage medium was not that critical. The best results for membrane integrity and sperm motility were obtained when the osmolarity of the storage medium was about 300 mOsm (De Pauw et al., 2003b). Besides their osmoregulating capacity, polyols and sugars are also used to stabilize
the spermatozoal membrane by replacing water molecules in the normally hydrated polar groups during transition through critical temperature zones (Woelders, 1997). Moreover, under aerobic conditions sorbitol can be used to a limited extent, as an appropriate metabolic substrate for spermatozoa (Clegg et al., 1986; Crichton et al., 1994). Notwithstanding the fact that sorbitol and mannitol are isomers, the small differences in structure seemed to affect their thermal protecting capacity.

When the effect of 5%, 10%, and 20% of egg yolk on the quality of the spermatozoa stored in CEP-2 diluent was evaluated, no significant differences were observed. Since CEP-2 diluent was developed for extended preservation of fresh bovine semen, and the fact that after 6 days of incubation the best motility results were obtained in CEP-2 diluent with 10% egg yolk, this concentration will be used in further experiments. Based on results obtained by Van Demark and Sharma (1957), diluents for bovine semen have included anywhere from 12.5% to 30% egg yolk. This is much more than in the Caprogen® diluent which is specifically developed for liquid storage of bovine semen at ambient temperature and contains only 5% of egg yolk (Shannon and Curson, 1982). However, no difference in fertility was observed when the egg yolk concentration was decreased from 20% to 5% (Shannon and Curson, 1983). In freezing media, egg yolk protects spermatozoa from cold shock and freezing damages. Synthetic liposomes and soya bean extracts have been evaluated as substitutes, but none of them were as good as egg yolk (De Leeuw et al., 1993; Van Wagtendonk-de Leeuw et al., 2000). In media for liquid storage of semen, the most important effect of egg yolk is probably binding of seminal plasma fractions that have a detrimental effect on survival of spermatozoa (De Pauw et al., 2003a, b). Inclusion of macromolecular substances and particularly egg yolk in media used for liquid semen storage has shown to have a positive effect on sperm survival (Shannon and Curson, 1972; Al-Somai et al., 1994; Prendergast et al., 1995).

In contrast to the Caprogen® diluent which is used for storage of fresh bovine semen under N₂ gas at 19°C, all experiments with our newly developed CEP-2 diluent were performed at 5°C. Due to the lower metabolism of the spermatozoa at 5°C than at 19°C (Salisbury and Van Demark, 1961), and 2) the lower risk of overgrowth by contaminants at 5°C than at 19°C (Yang and Chou, 2000), storage at this low temperature was preferred. Moreover, another study showed that sperm quality decreased more rapidly when spermatozoa were stored in CEP-2 diluent at 19°C than at 5°C (Verberckmoes et al., 2002b).
CONCLUSION

Given the correlation between progressive motility and in vivo fertilizing capacity (Zhang et al., 1998), and the high percentage of progressively motile spermatozoa (> 40%) up to 6 days of incubation in CEP-2 diluent with 1 mg/ml of sorbitol and 10% egg yolk indicate that this diluent is suitable for extended preservation of fresh bovine semen at 5°C. Whether these good in vitro results also can be reached in vivo still needs further investigation.
REFERENCES


Storage of fresh bovine semen in CEP-1 diluent


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Kirillov VS, Mozorov VA, 1936: Duration of survival of bull spermatozoa in an epididymis isolated from the testis. Anim Breed 5, 22.


CHAPTER 5

COMPARISON OF THREE DILUENTS FOR THE STORAGE OF FRESH BOVINE SEMEN

Theriogenology, 2004 in press.

Verberckmoes S., Van Soom A., Dewulf J., de Kruif A.
ABSTRACT

In New Zealand, 95% of the semen used for artificial insemination in cattle is processed as liquid semen. Storage of liquid semen for up to 3 days in Caprogen® diluent enables a tenfold reduction of the insemination dose, compared to frozen-thawed semen, without a reduction in fertility. In this Caprogen® diluent spermatozoa are stored under N2 gas in the presence of catalase. However, a new diluent (CEP-2), which was originally based on the biochemical composition of bovine cauda epididymal plasma, could become an appropriate alternative to Caprogen®.

In this study, the effect of addition of catalase to bovine spermatozoa stored for 6 days in CEP-2 diluent under aerobic and anaerobic conditions was evaluated and compared with a Tris diluent. Additionally, the quality and in vitro fertilizing capacity of fresh bovine semen stored for 6 days at 5°C in the Triladyl®, CEP-2 (without catalase and N2 gas) and Caprogen® diluent were compared.

Addition of 4.5 mg/ml catalase to CEP-2 diluent under aerobic and anaerobic conditions had no effect on sperm quality. Spermatozoa stored in CEP-2 diluent moved faster and straighter than spermatozoa stored in Triladyl® or Caprogen® diluent. The in vitro fertilization and polyspermy rates did not differ significantly between spermatozoa stored for 6 days at 5°C in CEP-2 and Caprogen® diluent, but were significantly lower for spermatozoa stored in Triladyl® diluent.

We can conclude that based on the in vitro results, the CEP-2 diluent is a better diluent than Triladyl® and a good alternative to the Caprogen® diluent for long term storage of fresh bovine semen at 5°C. To confirm these promising in vitro results further in vivo experiments are required.
INTRODUCTION

Before the optimization of the freezing and thawing procedure, artificial insemination (AI) in cattle was performed with fresh semen (Foote, 2002). Freshly ejaculated semen was diluted in an extender ranging from a simple salt solution to a more complex buffered medium (Foote, 2002). To facilitate long distance distribution of fresh semen, a shelf life of at least 3 days was required. First, a beneficial effect on the survival of fresh diluted semen was obtained by addition of egg yolk and buffering components such as phosphate, citrate, and Tris to the extender (Philips, 1939). Secondly, attempts were made to extend the life span of ejaculated spermatozoa by lowering the metabolic rate of the spermatozoa by decreasing the storage temperature to 5°C (Salisbury and Van Demark, 1961). Storage at 5°C not only extended the life span of spermatozoa, it also decreased the risk of overgrowth by contaminants (Yang and Chou, 2000). However, the use of frozen-thawed semen facilitates distribution of semen world wide and enables screening of bulls for infectious diseases before their semen is used in the field. These advantages have resulted in the fact that in the developed countries, except for New Zealand, most of the semen currently used for AI in cattle is processed as frozen-thawed semen (Chupin and Thibier, 1995). In rare cases when liquid semen is used for AI, it is most frequently stored in a Tris based diluent at 5°C. In New Zealand, the breeding season in cattle is very short and most of the inseminations are performed with fresh semen. The semen is collected, diluted and stored at ambient temperature under N_2 gas in the Caprogen® diluent. Originally, the Caprogen® diluent was developed for use at 5°C, but for practical reasons and due to good fertility outcomes after storage at 15°C to 27°C, it is now mostly used at ambient temperature (Shannon and Curson, 1984). The Caprogen® diluent typically contains catalase and is stored under N_2 gas. Catalase is added to the medium to minimise the level of peroxide generated in the storage medium (Shannon, 1968), while N_2 gas reduces the metabolic activity of the spermatozoa, and reduces the dissolved O_2 level in the medium (Shannon, 1964; Shannon, 1965). Bovine spermatozoa stored at ambient temperature (15 to 27°C) can be used for three days without a reduction in fertility rates when 2 million spermatozoa are used per insemination.

An alternative to the Caprogen® diluent could be the newly developed CEP-2 diluent, the composition of which was originally based on the biochemical composition of bovine Cauda Epididymal Plasma (CEP) (Verberckmoes et al., 2001). In chapter 5 it was shown that fresh bovine semen can be stored in this diluent for 6 days at 5°C. Even after 6 days of storage in
the CEP-2 diluent, more than 40% of the spermatozoa were still progressively motile. Since catalase and N₂ have a positive effect on the storage capacity of Caprogen® diluent, the effect of addition of catalase to CEP-2 diluent under aerobic and anaerobic conditions (under N₂ gas) was investigated. Additionally, the quality and in vitro fertilizing capacity of spermatozoa stored for 6 days at 5°C in CEP-2, Triladyl® and Caprogen® diluent were compared.

MATERIAL AND METHODS

Media

Chemicals and media were obtained from Sigma (Bornem, Belgium) and Life Technologies, Gibco BRL® products (Merelbeke, Belgium). Triladyl® (Minitüb, Tiefenbach, Germany), a commercial Tris based egg yolk diluent, was used for the dilution and coating of the ejaculated spermatozoa (De Pauw et al., 2003a). The storage media used in this study were: Tris, Triladyl®, CEP-2, and Caprogen® diluent (LIC, Hamilton, New Zealand). The composition of the different storage media is presented in Table 1, but the exact composition of the commercial diluents is not available for publication. Since the Caprogen® diluent has to be saturated with N₂ gas before use and saturation is achieved faster at low temperatures, the diluent was chilled and subsequently a gentle stream of N₂ gas was passed through it for 30 min. After reheating the saturated Caprogen® diluent to 30°C, spermatozoa were suspended and the airspace present in the storage tube was replaced by N₂ gas. Modified bicarbonate buffered TCM199 supplemented with 20% heat inactivated FBS (Hyclone-cat. No. SH30071.02) was used as culture medium, and IVF-TALP consisting of bicarbonate buffered Tyrode solution supplemented with BSA (6 mg/mL) and heparin (25 µl/mL) was used as fertilization medium.
Table 1: Composition of Tris and CEP-2 diluent.

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</table>

Collection and preparation of semen.

Fresh semen of dairy bulls with normal sperm quality (progressive motility, percentage membrane intact spermatozoa, and percentage spermatozoa with normal morphology; all parameters > 80%) was collected by means of an artificial vagina in a falcon tube (Becton and Dickinson®, France) containing 5 ml of Triladyl® diluent, in accordance with the method described by De Pauw et al. (2003). Immediately after collection, semen was centrifuged for 10 min at 720 x g, and then seminal plasma and Triladyl® diluent were removed. Finally, spermatozoa were suspended in the different media. The suspended semen was stored at a concentration of 25 x 10⁶/ml for 6 days at 5°C in air tight tubes.

Sperm quality assessment.

Sperm quality was determined before cooling (t0) and after 2 (t2), 4 (t4) and 6 days (t6) of storage. Sperm concentration was determined by means of a Bürker counting chamber and
light microscopy (x 200). Membrane integrity in the first experiment was determined by means of eosin-nigrosin staining and light microscopy (x 400). Spermatozoa with a white and red coloured head were defined as having intact and damaged plasma membranes, respectively. In the second experiment, the fluorescent SYBR-14 and propidium iodide staining (LIVE/DEAD® Sperm Viability Kit, Molecular Probes, Leiden, The Netherlands) and fluorescent microscopy (x 400) were used for the determination of the percentage of membrane intact (MI) spermatozoa. Membrane intact spermatozoa fluoresced green, while membrane damaged spermatozoa fluoresced red. Acrosome integrity was assessed by means of Pisum Sativum Agglutinin (PSA) staining with a Leica DMR fluorescence microscope (x 400) (Kawakami et al, 1993). Spermatozoa with a fluorescent green head indicated acrosome intact spermatozoa. Spermatozoa were considered as acrosome reacted when a fluorescent band was observed at the equatorial region. For the evaluation of the mitochondrial membrane potential, spermatozoa were labeled with the fluorescent JC-1 staining (Molecular Probes, Leiden, The Netherlands). This fluorophore exhibits potential-dependent accumulation in mitochondria, and can reversibly change its emission from green to orange with increasing transmembrane electrical potential. Sperm motility was determined subjectively by two independent trained persons by means of light microscopy (x 200) on a heated stage (37°C). A distinction was made between total motility (Tot. Mot.) and progressive motility (Prog. Mot.). Objective evaluation of sperm motility was performed on at least 1000 spermatozoa by means of a computer assisted sperm analyser (CASA) (Hamilton Thorn Ceros 12.1, Hamilton Thorne Research, Beverly, USA). Before assessment of sperm motility, samples were incubated for 5 min in open air at 37°C. Following this, five µL of each sperm aliquot was inserted in a Leja counting chamber at 37°C. The following instrument settings for the HTR Ceros 12.1 were: frames acquired, 30 at a frame rate of 60 frames/s; minimum contrast, 20; minimum cell size (pixels), 5; progressive cells: velocity average pathway (VAP), >50 µm/s; progressive cells: straightness, >60%. Spermatozoa with a VAP higher than 30 µm/s were considered motile. The sperm motility parameters determined with the Hamilton Thorn were: velocity average path (VAP), velocity straight line (VSL), velocity curved line (VCL), amplitude lateral head (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and percentage of progressively motile spermatozoa (PMS). The formula for straightness is \[ \text{VSL} / \text{VAP} \times 100 \], and for linearity: \[ \text{VSL} / \text{VCL} \times 100 \].
**In vitro fertilization.**

Cow ovaries were randomly harvested in a slaughterhouse and transported to the laboratory within 3 to 4 hours after collection. Follicles of 2 to 6 mm in diameter were aspirated using an 18-gauge needle and a 10 ml syringe. After being washed in Hepes buffered TALP medium, the cumulus-oocyte complexes were in vitro matured in 500 µl of maturation medium at 38.5°C in 5% CO₂ in air at 100% humidity for 24-26 hours. After maturation, oocytes were inseminated with a final sperm concentration of 1 x 10⁶ spermatozoa/ml in 500 µl of fertilization medium.

After fertilization, oocytes were vortexed for 3 min in 2 ml of Hepes-TALP to remove excess sperm and/or cumulus cells, fixed overnight in 2% paraformaldehyde and 2% glutaraldehyde in phosphate buffered saline (PBS) solution without calcium and magnesium, and subsequently stained for 10 min with 1 µg/ml of Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). The presumed zygotes were mounted in 100% glycerol and evaluated by means of a Leica DMR fluorescence microscope for fertilization and polyspermy. Successful fertilization was characterized by the presence of two pronuclei (normal fertilization). The presence of more than two pronuclei indicated polyspermy.

**Experimental design.**

**Experiment 1: Effect of catalase under aerobic and anaerobic conditions on the storage capacity of the CEP-2 diluent.**

Fresh semen of 3 bulls was collected and diluted in airtight Falcon® tubes containing 4 storage media: 1) CEP-2 diluent, 2) CEP-2 diluent + 4.5 mg/ml catalase (aerobic), 3) CEP-2 diluent + 4.5 mg/ml catalase + N₂ (anaerobic), and 4) Tris diluent. The Tris diluent was used as control medium. The diluted semen was slowly cooled to 5°C and stored for 6 days. Membrane and acrosome integrity, and sperm motility (objectively and subjectively) were evaluated every two days.

The experiment was repeated 4 times and the results were analysed using the linear mixed effect model with the bull as random variable and an autoregressive correlation structure of the first order (S-Plus, 2000 Math Soft Inc).
Experiment 2: Comparison of sperm quality parameters after storage in Triladyl®, CEP-2, and Caprogen® diluent.

Fresh semen of 4 bulls was collected as described before and stored in Triladyl®, CEP-2 and Caprogen® diluent for 6 days at 5°C. Membrane and acrosome integrity, and sperm motility (objectively and subjectively) were evaluated every two days. The mitochondrial membrane potential of the spermatozoa was evaluated after 6 days of storage in the different diluents. The experiment was repeated 3 times and the results were analysed using the linear mixed effect model with the bull as random variable and an autoregressive correlation structure of the first order (S-Plus, 2000 Math Soft Inc).

Experiment 3: Comparison of in vitro fertilizing (IVF) capacity of spermatozoa stored for 6 days in Triladyl®, CEP-2, and Caprogen® diluent.

Semen of a bull with good sperm quality was collected, prepared and stored in Triladyl®, CEP-2 and Caprogen® diluent. After 6 days of storage, spermatozoa were centrifuged for 10 min at 720 x g, separated on a discontinuous Percoll® gradient and live spermatozoa were subsequently used for IVF. To verify if the experiment was affected by external factors, a control group was included in which IVF was performed with frozen-thawed semen of a bull with proven IVF rates. For each replicate, matured oocytes were divided in four equal groups and each group was randomly inseminated with semen of one of the four treatments.

The experiment was repeated 4 times and the results were analyzed using a logistic regression model with Caprogen® as reference (SPSS 11.0, 2002).

RESULTS

Experiment 1: Effect of catalase under aerobic and anaerobic conditions on the storage capacity of the CEP-2 diluent.

Except for membrane integrity (p = 0.57), ALH (p = 0.06), and STR (p = 0.43), a significant (p < 0.01) decrease over time of all evaluated sperm quality parameters was observed in all media. For membrane integrity, progressive motility, VAP, VSL (Figure 1a), ALH, BCF (Figure 1b), STR and LIN, a significant (p < 0.05) difference was found between the different media. However, between the different media no significant difference in total motility (p = 0.39) and PMS (p = 0.12) (Figure 1c) was found. Except for ALH, the highest sperm quality values were obtained for spermatozoa stored in different CEP-2 diluents. The
ALH values were significantly ($p < 0.01$) higher in Tris diluent than in different CEP-2 diluents. These results indicate that spermatozoa stored in the different CEP-2 diluents moved faster and in a straighter line than spermatozoa stored in Tris diluent. Moreover, the higher ALH values in Tris diluent are indicative of hyperactivation of spermatozoa.

Between spermatozoa stored in CEP-2 diluent, CEP-2 diluent + catalase (aerobic), and CEP-2 diluent + catalase + $N_2$ (anaerobic), no significant difference in membrane integrity and sperm motility parameters was observed ($p > 0.05$). The acrosome integrity at t6 was not significantly different between the media and ranged between 94% and 97% ($p = 0.54$).

**Experiment 2: Comparison of sperm quality parameters after storage in Triladyl®, CEP-2, and Caprogen® diluent**

The percentage of membrane and acrosome intact spermatozoa significantly ($p < 0.01$) decreased over time from 79.4 $\pm$ 13.4% and 98.2 $\pm$ 0.8% at t0, to 60.8 $\pm$ 16.1% and 94.7 $\pm$ 2.2% at t6 respectively. Between diluents, no significant difference in membrane ($p = 0.52$) and acrosome ($p = 0.48$) integrity was observed.

After 6 days of storage, the percentage of spermatozoa with a high mitochondrial membrane potential did not differ between spermatozoa stored in CEP-2 (97.8 $\pm$ 2.8%) and
Caprogen diluent (99.3 ± 1.5%) (p = 0.74), while it was significantly (p < 0.01) lower for spermatozoa stored in Triladyl® diluent (80.0 ± 19.0%).

Total and progressive motility (subjectively evaluated) decreased significantly over time (p < 0.01), but no significant differences were found between different diluents (p = 0.18 and p = 0.12 respectively) (Figure 2a). After 2 to 4 days of storage, the percentage of motile spermatozoa in Triladyl® and Caprogen diluent which showed extremely vigorous and flagellar movements ranged between 65% and 90%, and were defined as being hyperactivated. Except at day 2 (18%), no hyperactivated spermatozoa were detected in CEP-2 diluent during the 6 days of storage.

![Figure 2: Progressive motility of spermatozoa stored for 6 days at 5°C in Triladyl®, CEP-2, and Caprogen diluent subjectively (a) and objectively (b) determined.](image)

When sperm motility was objectively assessed by means of the Hamilton Thorne, a significant (p < 0.01) decrease of all evaluated sperm motility parameters over time was observed except for ALH (p = 0.06). Between diluents, significant differences (p ≤ 0.01) were obtained for ALH, BCF, STR and LIN, although not for VAP (p = 0.25), VCL (p = 0.28), PMS (p = 0.62) (Figure 2b), and VSL (p = 0.06). Straightness (STR) and VSL values were significantly higher (p < 0.01) in CEP-2 than in Triladyl® and Caprogen® diluents, whereas BCF and LIN values in CEP-2 diluent were not significantly higher than in Triladyl® and Caprogen® diluent. The higher values for VSL, BCF, STR and LIN for spermatozoa stored in CEP-2 diluent indicate that they move faster and straighter than spermatozoa stored in other diluents (Figure 3). The significantly higher values for ALH that have been obtained for spermatozoa stored in Caprogen® diluent indicate an increased proportion of hyperactivated spermatozoa.
Figure 3: Velocity straight line (VSL) (a), beat cross frequency (BCF) (b), and linearity (LIN) (c) of spermatozoa stored for 6 days at 5°C in Triladyl®, CEP-2, and Caprogen diluent determined by means of Hamilton Thorne.

**Experiment 3: Comparison of in vitro fertilizing (IVF) capacity of spermatozoa stored for 6 days in Triladyl®, CEP-2, and Caprogen® diluent.**

No significant differences in fertilization and polyspermy rates were observed between fresh semen stored in CEP and Caprogen® diluents, and frozen-thawed semen (= control group). However, after storage in Triladyl® diluent, fertilization and polyspermy rates decreased significantly in comparison with Caprogen® (p < 0.01) (Table 2).

Table 2: Fertilization, penetration and polyspermy rate of fresh bovine semen after 6 days of storage at 5°C in CEP-2, Caprogen® and Triladyl® diluent, and of frozen-thawed semen of a bull with proven IVF rates (control).

<table>
<thead>
<tr>
<th></th>
<th>Oocytes (N)</th>
<th>Normal fertilization (%)</th>
<th>Polyspermy (%)</th>
<th>Penetration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP-2</td>
<td>192</td>
<td>96 (50.0%)^a</td>
<td>22 (11.5%)^a</td>
<td>118 (61.5%)^a</td>
</tr>
<tr>
<td>Caprogen®</td>
<td>211</td>
<td>109 (51.7%)^a</td>
<td>31 (14.7%)^a</td>
<td>140 (66.4%)^a</td>
</tr>
<tr>
<td>Triladyl®</td>
<td>207</td>
<td>45 (21.7%)^b</td>
<td>6 (2.9%)^b</td>
<td>51 (24.6%)^b</td>
</tr>
<tr>
<td>Control</td>
<td>182</td>
<td>105 (57.7%)^a</td>
<td>31 (17.0%)^a</td>
<td>136 (74.7%)^a</td>
</tr>
</tbody>
</table>

^a,b values within the same column are significantly different (P < 0.01).
DISCUSSION

In this study, comparable results were obtained for the motility parameters of spermatozoa stored for 6 days in CEP-2, Caprogen®, and Triladyl® diluent. IVF results were significantly better after 6 days of storage in CEP-2 and Caprogen® diluent compared to storage in Triladyl® diluent. To our knowledge, the newly developed CEP-2 diluent is the first medium which can be used as full alternative to the Caprogen® diluent for prolonged preservation of fresh bovine semen.

The extended lifespan of spermatozoa in Caprogen® diluent can largely be attributed to the presence of catalase in the medium and the storage under anaerobic conditions (Shannon, 1964, 1965, 1973). The protective effect of catalase on membrane integrity of spermatozoa is based on the reduction of the levels of peroxide generated in the storage medium. The production of reactive oxygen species in aerobic as well as partially aerobic systems is inevitable (Mann and Lutwak-Mann, 1975, 1981). The main source of peroxide production during storage of bovine spermatozoa at ambient temperature is the oxidative deamination of aromatic amino acids by a membrane-bound aromatic amino acid oxidase (AAAO). The AAAO enzyme is heat and acid labile and is restricted to dead spermatozoa (Shannon and Curson, 1972). The storage under N₂ gas reduces the oxygen tension and production of reactive oxygen species (ROS), without affecting the pH of the medium. However, N₂ gas could substantially reduce the metabolic activity of spermatozoa (Shannon, 1964, 1965). In contrast to Caprogen®, addition of catalase to CEP-2 diluent had no beneficial effect on sperm quality, even under anaerobic conditions. Storage at 5°C may not only decrease the production of ROS, but also the enzymatic activity of catalase. The CEP-2 diluent is developed for storage of bovine semen at 5°C, while the Caprogen® can be used at 5°C, but proved to be better for semen stored at ambient temperature (Mann and Lutwak-Mann, 1981). Since spermatozoa were stored at 5°C in the present experiment, less reactive oxygen species (ROS) might have been produced due to the reduced activity of the AAAO enzyme, which is temperature dependent (Shannon and Curson, 1972; 1982a, 1982b). Decreased storage temperatures do not only decrease the metabolic activity of spermatozoa and ROS producing enzymes, but also decrease the activity of the Na / K pump which copes with the diffusion of ions across the cell membrane (Sweedner and Goldin, 1980). However, the specific ionic composition and ion ratios in the CEP-2 diluent may counteract this problem, since membrane
Comparison of three diluents for the storage of fresh bovine semen

and acrosome integrity, and the different sperm motility parameters, remained very good during the 6 days of storage. This is in contrast to the spermatozoa stored in Tris diluent. Since progressive motility is one of the most reliable parameters to predict the fertilizing capacity of bovine spermatozoa (Zhang et al., 1998; Correa et al., 1997; Verberckmoes et al., 2002), special attention was paid to the different motility parameters. The progressive motility of spermatozoa stored in CEP-2 diluent decreased slowly from 79.4 ± 13.4% before incubation to 60.8 ± 16.1% after 6 days of incubation at 5°C, independent of the presence of catalase and N2 in the medium. The motile spermatozoa in CEP-2 diluent also showed a remarkably good straight line motility, indicated by the high values for straightness (>80%) and linearity (>50%) during the whole storage period. The velocity straight line (110 µm/s) and the beat cross frequency (35 Hz) of spermatozoa stored in CEP-2 diluent were twice as high as those of spermatozoa stored in Tris diluent for 6 days. The increased amplitude of lateral head displacement of spermatozoa stored in Tris diluent indicated that more spermatozoa were hyperactivated than in CEP-2 diluent.

In the second experiment, the storage capacity of CEP-2 diluent was compared with two other diluents which have been used for long term preservation of fresh bovine semen: Triladyl® (De Pauw et al., 2003b) and Caprogen® (Shannon and Curson, 1984). No significant differences in membrane and acrosome integrity or in mitochondrial membrane potential were observed between the three diluents during the 6 days of storage. However, spermatozoa stored in CEP-2 diluent moved significantly faster (VSL, BCF) and straighter (STR, LIN) than in Triladyl® and Caprogen® diluent. When sperm motility was evaluated subjectively, increased percentages of hyperactivated spermatozoa were observed in Caprogen® and Triladyl® diluents. This finding was confirmed by the increased amplitudes for lateral head displacement (ALH) that were obtained with the Hamilton Thorne.

Although membrane intact spermatozoa can be motile for an extended period of time, it is not obvious that they are still capable of fertilizing. In the Illini Variable Temperature (IVT) diluent e.g., spermatozoa can be stored under CO2 gas for over 90 days at 5°C while maintaining their motility (> 45%), but after 2 days of storage the fertilizing capacity decreases sharply (Bartlett and Van Demark, 1962). In this study, in vitro fertilization rates of 50% or more were obtained with frozen-thawed spermatozoa and with spermatozoa stored for 6 days at 5°C in CEP-2 and Caprogen® diluent. Significantly lower fertilization rates were obtained with spermatozoa stored in Triladyl® diluent. These results indicate that spermatozoa stored either in CEP-2 or in Caprogen® diluent have the same in vitro fertilizing capacity after
6 days of storage. To determine if the in vivo fertilizing capacity of spermatozoa stored in CEP-2 diluent is as good as that of spermatozoa stored in Triladyl® or Caprogen® diluent, more research in the field has to be performed.

In conclusion, the comparable sperm motility and IVF results between spermatozoa stored for 6 days in CEP-2 and Caprogen® diluent, indicate that the newly developed CEP-2 diluent can provide an alternative for prolonged preservation of fresh bovine semen. In vivo experiments are required to confirm the in vitro results.
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MIGRATION OF BOVINE SPERMATOZOA IN A SYNTHETIC MEDIUM AND ITS RELATION TO IN VIVO BULL FERTILITY

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Verberckmoes S, Van Soom A, De Pauw I, Dewulf J, de Kruif A
ABSTRACT

A great deal of research has been performed to predict the in vivo bull fertility by means of a simple in vitro test. One of such test is the sperm migration test which has been extensively evaluated in human infertility studies. However, its application to predict bovine fertility has been very limited, and a clear relation between the sperm migration distance and in vivo bull fertility has never been demonstrated. A synthetic medium based upon methyl cellulose (MC) was tested for its suitability to serve as a migration medium for frozen-thawed bovine spermatozoa. The effects of the concentration of MC, the incubation time, and sperm concentration on sperm migration capacity was determined. The relation between sperm migration capacity at different incubation times of the frozen-thawed spermatozoa of five bulls, and their 56 d non-return rates (NRRs) was assessed in order to evaluate its suitability as a tool to predict in vivo bull fertility.

The highest repeatability of the sperm migration test (CV = 10.7%) was obtained when the sperm migration distance of the five vanguard motile spermatozoa was determined at 30 min incubation at 37°C in a migration medium with 1.35% MC. No significant difference in migration distance was demonstrated when sperm concentrations of 100 x 10^6 and 150 x 10^6 spermatozoa/ml were used. Despite the relatively high repeatability of the migration test, no relation was found between the sperm migration distance and the 56d-NRRs of five sire bulls. Therefore, the sperm migration test in 1.35% MC cannot be used to predict in vivo bull fertility accurately.
INTRODUCTION

During natural mating, spermatozoa must migrate through the cervical mucus to enter the uterus. Subsequently, spermatozoa are transported mainly by muscular movements of the uterus to the functional sperm reservoir in the oviduct (Suarez et al., 1990). It is obvious that when spermatozoa cannot reach the site of fertilization for any reason, they will be unable to make use of their potential to fertilize (Hunter, 1987; Katz et al., 1989).

To study spermatozoal motility potential, physical barriers to be overcome in vivo can be mimicked by cervical mucus in sperm migration assays. Cervical mucus from human females (Reichmann et al., 1973) and from cows (Kummerfeld et al., 1981) has been used for sperm migration assays to evaluate the fertility of human semen donors in vitro. In humans, a number of independent studies have shown that the fertilizing capacity of spermatozoa is correlated with penetration of cervical mucus (Alexander, 1981; Hull et al., 1984). However, sperm migration distances are influenced by the mucus donor and by the length of time the mucus was stored before testing (Kummerfeld et al., 1981; Lee et al., 1981). In addition, there might be technical difficulties with filling capillary tubes with cervical mucus (Tang et al., 1999). The use of a substitute for cervical mucus can overcome the problem of characteristic changes in the cervical mucus over time (Aitken et al., 1992). Alternatives that have been tested in sperm migration assays are hen egg white (Eggert-Kruse et al., 1990; Bostofte et al., 1992; Engel and Petzoldt, 1999), hyaluronic acid (Neuwinger et al., 1991; Mortimer et al., 1990; Aitken et al., 1992) and polyacrylamide gel (Lorton et al., 1981; Goldstein et al., 1982; Parrish and Foote, 1987; Eggert-Kruse et al., 1993). Although sperm migration has been extensively refined and validated in human infertility studies (Clarcke et al., 1998; Biljan et al., 1994), its application to predict bovine fertility has been very limited and a clear relation between the sperm migration distance and in vivo bull fertility has never been demonstrated (Kummerfeld et al., 1981; Matousek et al., 1989).

The aim of this study was to test a simple sperm migration assay for cattle, which can be performed easily under field conditions. For this purpose, a synthetic medium based upon methyl cellulose was evaluated for its suitability as a sperm migration medium. Methyl cellulose is an unbranched chain polymer and has been extensively used to test effects of viscosity on sperm movements (Pate and Brokaw, 1980; Suarez and Dai, 1992). The repeatability of the sperm migration test was optimised and the relation between bovine
fertility as assessed by 56d non-return rates (NRRs) and the migration distance in this synthetic medium was investigated.

MATERIAL AND METHODS

General Material and Methods

Synthetic migration medium. The synthetic media consisted of a mixture of Hepes-TALP and methyl cellulose (MC) of 1500 centipoise (cps) (Federa®, Brussels, Belgium). The final solutions contained 0.27%, 0.68% and 1.35% MC. Solutions were homogenized by shaking, and stored at 4°C up to 1 week before use. The pH, osmolarity and viscosity of the three media were determined. Their pH was determined at 20°C by means of a pH meter (pH 526, TUV®, Weilheim, Germany) and their osmolarity was assessed by determining the freezing point with an osmometer (Fisk Associates®, Massachusetts, USA). The viscosity of the media was determined at 37°C by means of the Haake PG 142 viscometer (Haake®, Karlsruhe, Germany) at four rotation speeds: 64, 128, 256 and 512 rpm (Table 1).

Table 1. pH, osmolarity and viscosity of migration medium 1, 2 and 3 with 0.27%, 0.68% and 1.35% methyl cellulose (MC) respectively, determined at four rotation speeds.

<table>
<thead>
<tr>
<th>Concentration of MC</th>
<th>pH</th>
<th>Osmolarity</th>
<th>Viscosity (mPas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 1</td>
<td>0.27 %</td>
<td>7.1</td>
<td>295</td>
</tr>
<tr>
<td>Medium 2</td>
<td>0.68 %</td>
<td>7.1</td>
<td>289</td>
</tr>
<tr>
<td>Medium 3</td>
<td>1.35 %</td>
<td>7.1</td>
<td>287</td>
</tr>
</tbody>
</table>

Treatment of spermatozoa. Frozen bull semen was provided by the Flemish Artificial Insemination Centre (VRV). The ejaculates were diluted in an egg yolk-Tris-glycerol extender. The semen was frozen in 0.25 ml straws and maintained in liquid nitrogen at –196°C until use. Frozen semen of the same batch was used throughout the experiment. To determine the repeatability of the sperm migration assay, frozen semen of six bulls was used. The frozen-thawed semen used for the determination of the NRRs originated from the same batch as the semen used for the sperm migration test.

Sperm migration test. The in vitro migration apparatus consisted of non-heparinised round microhaematocrite capillaries (Hirschmann®, Eberstadt, Germany) which were fixed in plastic petridishes to facilitate handling. In combination with inverted microscopy, round
capillaries are as suitable as specially designed flat tubes (Kummerfeld et al., 1981). The length of the capillaries was 7.5 cm and they had an internal diameter of 1.5 mm. They were filled with the selected migration medium and closed at one end with putty. At the other end, 10 µL of space was left in which the sperm suspension was injected. The capillaries were pre-warmed in a humidified chamber at 37°C. Only capillaries free of air bubbles were used. At time zero (t0), 10 µL of sperm suspension was injected in the open end of the capillary. The migration distance was determined at different time intervals after injection by means of an inverted light microscope (Olympus® IX70-S8F, Japan) equipped with a heating stage at 37°C at magnification of x 200 (Figure 1). The capillaries were examined microscopically from the end where the sperm was injected to the most distant point containing motile spermatozoa. Since we were interested in spermatozoa with a good migration capacity, the “migration distance” for the vanguard one and five spermatozoa (including the vanguard one) was measured (millimetres). The migration distance of the vanguard spermatozoa was measured from the place of injection to the farthest point of migration.

![Figure 1. Determination of sperm migration distance in vitro by means round microhaematocrite capillaries (Hirschmann®, Germany), which were fixed in plastic petridishes to facilitate handling, in combination with inverted microscopy.](image)

**Experimental Design and Statistical Analysis**

**Experiment 1: Influence of methyl cellulose concentration on the sperm migration distance.**

In this experiment, for each concentration of methyl cellulose (0.27, 0.68 and 1.35% MC) six replicates per bull were used. The test was performed with frozen-thawed semen of six bulls. After thawing the semen, one ml of Hepes-TALP was added and the sperm suspension was centrifuged for 5 min at 514 x g. The supernatant was removed after centrifugation and the spermatozoa were diluted to obtain a final concentration of 120 x 10^6 spermatozoa/ml.
Sperm quality was determined before injection into the capillary tubes for the sperm migration test. The migration distance was determined for the one and five vanguard motile spermatozoa at 15 and 30 min after injection of the spermatozoa into the capillary tubes and incubation in the humidified chamber at 37°C. The comparison of the different media and different spermatozoa concentrations in relation to the sperm migration distance was done by using ANOVA (SPSS 10, SPSS Inc. Illinois 60611, USA, 1999).

Experiment 2: Repeatability of the sperm migration test.

Semen from six bulls was used to measure migration in 1.35% MC. Per bull, six replicates of the sperm migration test were performed. In each capillary 120 x 10⁶/ml of spermatozoa were injected and the migration distance of the one and five vanguard motile spermatozoa was determined at 15 (t1), 30 (t2), 45 (t3) and 60 min (t4) after incubation in the humidified chamber at 37°C. The repeatability of the sperm migration test was determined by evaluating the coefficient of variation for the one and five vanguard motile spermatozoa.

Experiment 3: Influence of sperm concentration on the sperm migration distance.

Semen from two bulls was used to perform the sperm migration assay in a medium with 1.35% methyl cellulose. The frozen-thawed semen was centrifuged for 5 min at 514 x g and concentrated to 100 x 10⁶ and 150 x 10⁶ spermatozoa/ml. For each concentration, six replicates per bull were tested. In contrast to the previous experiments, the migration distance was not determined by assessing the migration point of the one and five vanguard spermatozoa but was determined by the most distant point containing five motile spermatozoa per microscopic field at t0 (moment of injection), t1 (15 min) and t2 (30 min) after injection of the spermatozoa. The influence of the two sperm concentrations (100 x 10⁶ and 150 10⁶/ml) on the sperm migration distance was determined by means of the independent sample t-test (SPSS 10, SPSS Inc. Illinois 60611, USA, 1999).

Experiment 4: Relation between 4 sperm quality parameters and their predictive value for NRRs.

Frozen-thawed semen from five bulls with NRRs ranging from 52.8% to 66.7% was used to determine progressive motility, membrane integrity, sperm concentration and the sperm migration distance. The spermatozoa used for the sperm migration assay were of the same batch as those used for the determination of in vivo fertility. The NRRs were determined after 219 ± 16 (mean ± SD) first inseminations in heifers and cows. The inseminations were performed 12 to 18 h after the first estrous signs observed by the farmer. The NRRs were not
known by the investigators at the time the sperm migration experiments were performed. Prior to freezing, the semen from the five bulls exhibited minimum values of 80% progressive motility, 80% membrane intact spermatozoa and a concentration of at least $0.75 \times 10^9$ spermatozoa/ml. After thawing, the percentage of progressive motile and membrane intact spermatozoa and sperm concentration ranged from 25 to 50%, 62 to 86% and 6 to $14 \times 10^6$ spermatozoa/ml respectively for the five bulls.

The progressive motility of the bull semen was evaluated subjectively at 37°C. Eosin-nigrosin staining was used for the assessment of membrane integrity. The sperm concentration was determined by means of a Bürker counting chamber. These three basic parameters were evaluated by means of light microscopy. The sperm migration capacity was determined in the Hepes-TALP medium containing 1.35% methyl cellulose. Six replicates of the sperm migration assay were performed per bull. The migration distance of the five vanguard spermatozoa was measured at 15 min (t1) and 30 min (t2) after injecting $120 \times 10^6$ spermatozoa/ml. The correlation between the sperm migration distance at t2 and the three other sperm quality parameters, as well as the correlation between the NRRs of the five bulls, and the progressive motility, membrane integrity and sperm migration distance at t2, was determined by calculating the correlation coefficient (R). Linear regression was used to evaluate the predictive properties of the 4 sperm quality parameters on in vivo fertility (NRR), as well as to evaluate the relation between the sperm migration distance and the three other sperm quality parameters (SPSS 10, SPSS Inc. Illinois 60611, USA, 1999).

**RESULTS**

*Experiment 1: Influence of methyl cellulose concentration on the sperm migration distance.*

The migration distance of the one and five vanguard spermatozoa after 15 min of incubation was significantly shorter in the medium with the highest viscosity than in the two other media. After 30 min of incubation, the migration distance for the one and five vanguard motile spermatozoa was significantly different for the three media (P<0.05) (Table 2). The most viscous medium (1.35% MC) yielded the lowest mean sperm migration distance.
Table 2. Migration distance in mm (Mean ± SEM.) for 6 replicates per bull (n = 6) of the vanguard one and five motile spermatozoa after 15 and 30 min incubation in migration medium with three different concentrations of methyl cellulose (MC).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Migration distance at 15 min</th>
<th>Migration distance at 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vanguard one</td>
<td>vanguard five</td>
</tr>
<tr>
<td>0.27 % MC</td>
<td>30.9 ± 0.23\textsuperscript{a}</td>
<td>26.8 ± 0.19\textsuperscript{a}</td>
</tr>
<tr>
<td>0.68 % MC</td>
<td>28.6 ± 0.23\textsuperscript{a}</td>
<td>23.6 ± 0.19\textsuperscript{a}</td>
</tr>
<tr>
<td>1.35 % MC</td>
<td>18.3 ± 0.23\textsuperscript{b}</td>
<td>16.0 ± 0.19\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b,c,d,e} Values with different superscripts in the same column are significantly different (P < 0.05).

**Experiment 2: Repeatability of the sperm migration test.**

From experiment 2, only medium 3 was used for the sperm migration assay. The repeatability of the sperm migration assay in the medium with 1.35% MC for all six bulls is presented in Table 3. The repeatability of the sperm migration assay increased with increasing incubation time and was higher for the five vanguard spermatozoa than for the one vanguard spermatozoon. It was noticed that the number of motile spermatozoa decreased substantially after > 30 min incubation at 37°C. Therefore, the sperm migration at a later point of time was not considered reliable despite the low CV. From table 3 it is clear that the highest repeatability at t2 is obtained for the 5 vanguard spermatozoa (CV = 10.7 ± 3.7).

Table 3. Repeatability of the sperm migration assay determined by the coefficient of variation (CV) for the vanguard one and five motile spermatozoa in migration medium with 1.35% methyl cellulose.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vanguard one spermatozoon</td>
</tr>
<tr>
<td>15 min (t1)</td>
<td>11.4</td>
</tr>
<tr>
<td>30 min (t2)</td>
<td>11.0</td>
</tr>
<tr>
<td>45 min (t3)</td>
<td>10.0</td>
</tr>
<tr>
<td>60 min (t4)</td>
<td>8.1</td>
</tr>
<tr>
<td>Average</td>
<td>10.0</td>
</tr>
</tbody>
</table>

**Experiment 3: Influence of sperm concentration on the sperm migration distance.**

Although the five vanguard spermatozoa at t1 and t2 of incubation showed a tendency to migrate more at a concentration of 150 x 10\textsuperscript{6} spermatozoa/ml than at a concentration of 100 x 10\textsuperscript{6} spermatozoa/ml, this difference was only significant at t1 (P = 0.89 at t0, P = 0.02 at t1, P = 0.06 at t2) (Figure 2).
Experiment 4: Relation between 4 different sperm quality parameters and their predictive value for NRRs.

As is shown in Table 4, sperm concentration (P < 0.01) and membrane integrity (P = 0.05) were significantly related to the sperm migration distance at t2. Moreover, the sperm concentration could explain 64% of the variation in migration distance. The correlation between progressive motility and the sperm migration distance was very low (r = 0.05). As is presented in Table 5, it is obvious that the progressive motility was the sperm quality parameter which is most correlated (r = 0.66, P < 0.01) to the NRRs of the five bulls. The sperm migration distance at t1 and t2 was also significantly related to the NRRs, albeit in a negative way (β1 -0.50 and -0.43, respectively). The correlation between membrane integrity and NRRs was negligible (r = 0.05).

Table 4. Relation between progressive motility, membrane integrity, sperm concentration and sperm migration distance of bovine spermatozoa was tested at 30 min of incubation in a migration medium of 1.35% methyl cellulose.

<table>
<thead>
<tr>
<th>Sperm migration distance at t2</th>
<th>β0</th>
<th>β1</th>
<th>p</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive motility</td>
<td>36.48</td>
<td>-0.02</td>
<td>0.81</td>
<td>0.05</td>
</tr>
<tr>
<td>Membrane integrity</td>
<td>26.27</td>
<td>0.14</td>
<td>0.05</td>
<td>0.36</td>
</tr>
<tr>
<td>Concentration</td>
<td>22.00</td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Linear regression was used to determine the regression coefficients (β0 and β1) and the correlation coefficient (r).
Table 5. Use of sperm quality parameters: progressive motility, membrane integrity, migration distance after 15 min \((t1)\) and 30 min \((t2)\) incubation in a medium of 1.35% methyl cellulose to predict non return rate (NRR).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(\beta_0)</th>
<th>(\beta_1)</th>
<th>(p)</th>
<th>(r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive motility</td>
<td>49.32</td>
<td>0.32</td>
<td>&lt;0.01</td>
<td>0.66</td>
</tr>
<tr>
<td>Membrane integrity</td>
<td>58.19</td>
<td>0.02</td>
<td>0.81</td>
<td>0.05</td>
</tr>
<tr>
<td>Migration distance at (t1)</td>
<td>74.75</td>
<td>-0.50</td>
<td>&lt;0.01</td>
<td>0.52</td>
</tr>
<tr>
<td>Migration distance at (t2)</td>
<td>74.69</td>
<td>-0.43</td>
<td>0.02</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Linear regression was used to determine the regression coefficients \((\beta_0 \text{ and } \beta_1)\) and the correlation coefficient \((r)\).

**DISCUSSION**

The capacity for sperm migration through cervical mucus depends on the structural–mechanical properties of the mucus microstructure, the viscosity of the mucus plasma and the size and shape of the sperm head and flagellum (Katz et al., 1989). In this study, we used a synthetic medium based upon MC instead of cervical mucus to obtain a sperm migration medium that could be tested for its suitability to predict in vivo bull fertility. Cervical mucus of estrous cattle has a pH of about 7, a viscosity of 23.2 mm H\(_2\)O, and a Spinnbarkeit of 72 mm. Spermatozoa can migrate in cervical mucus on average a distance of 13 mm for the first 10 min (Tsiligianni et al., 2001). The migration medium with the highest concentration of MC (1.35% MC) had a pH of 7.1, and spermatozoa migrated a distance of 16 mm within 15 min, which is very similar to the values obtained in bovine cervical mucus. However, the Spinnbarkeit of our MC medium was only 7 mm, and unlike polyacrylamide or cervical mucus, it did not have the appearance of a gel. The 1.35% MC medium had a viscosity of 65.5 mPas but because of the difference in units, it is difficult to compare the viscosity of the MC medium with that of cervical mucus. From Experiment 1 it is obvious that sperm movement was depressed when the viscosity of the sperm migration medium increased. Since the sperm migration capacity in the 1.35% MC medium and cervical mucus were relatively similar, and the length of the capillary tubes was not a limiting factor for sperm migration capacity in the 1.35% MC medium, this medium was used for further experiments. Methyl cellulose is an unstructured and unreactive buffer that interferes with sperm migration only because of its increased viscosity and was, therefore, perfectly suited for our purpose, which was to evaluate sperm migration through a synthetic medium with good repeatability. Sperm migration in a MC solution was quite repeatable as indicated by a CV of 10.7%. Sperm
samples which were introduced into the capillary at two different concentrations showed a tendency to migrate further at a concentration of 150 x 10^6/ml than at a concentration of 100 x 10^6/ml, although this was only significant at t1 (P = 0.02). This tendency was confirmed in Experiment 4 where concentration had a significant positive correlation with the sperm migration distance.

In a number of studies, sperm migration capacity has been linked with other sperm characteristics such as total motility, sperm morphology, progressive motility, acrosome integrity and sperm concentration (Hull et al., 1984; Galli et al., 1991). In our study, only the correlations between sperm migration capacity and progressive motility, membrane integrity and sperm concentration were determined. In a recent study, Anilkumar et al. (2001) found that sperm penetration in polyacrylamide gel and bovine cervical mucus was positively correlated with post-thaw motility (r = 0.81; r = 0.89: P < 0.01) and acrosome integrity (r = 0.88; r = 0.94: P < 0.01) of bovine spermatozoa. However, in contrast to this (Anilkumar et al., 2001) and other studies (Goldstein et al., 1982; Galli et al., 1991), in our study no correlation was found between progressive motility and the sperm migration distance (r = 0.05, P = 0.81). In accordance with Galli et al. (1991) and Goldstein et al. (1982) we did found a positive correlation between the sperm migration distance and membrane integrity (r = 0.36, P = 0.05) and concentration (r = 0.64, P < 0.01).

Although sperm migration capacity has been linked with different sperm quality parameters, it has seldom been linked with in vivo fertility in ruminants (Murase et al., 1990). In contrast to studies in humans (Alexander, 1981; Goldstein et al, 1982), we were not able to establish a positive correlation between the sperm migration distance in a migration medium with 1.35% MC and the NRRs of five proven sires. The sperm migration distance at t1 and t2 was even negatively related (β1 -0.50 and -0.43 respectively, P < 0.01 and 0.02 respectively) to the NRRs. In contrast to this, bull fertility and the sperm migration distance were linked in a positive way in a previous study (Murase et al., 1990). However, the experimental design in that study differed from ours in two ways: first, the sperm migration assay was performed in cervical mucus, and second, fresh spermatozoa were used, whereas the NRRs were obtained after insemination with frozen-thawed spermatozoa. This means that different ejaculates were used for the sperm migration assay and for the determination of the NRRs, whereas in our study the frozen-thawed semen used for the sperm migration test and for the determination of the NRRs originated from the same batch.
Apart from the experimental design there may be several explanations for the disparity in results between different bovine studies and between bovine and human experiments. First, it could be due to differences in characteristics between the media used. Despite the fact that the MC medium yielded repeatable results it might not be as effective as hyaluronate, cervical mucus or polyacrylamide gel for sperm migration tests. Hyaluronate and cervical mucus possess a particular alignment of mucin chains resulting in a higher viscosity and in sperm movement parallel to the axis of the tube into mucus and hyaluronate solution channels (Tang et al., 1990). In contrast to cervical mucus, the sperm penetrability in synthetic media such as MC and hyaluronate is not impaired by the presence of anti-sperm antibodies (Tang et al., 1990). Polyacrylamide has proved to be more effective than MC when it comes to favouring migration of hyperactivated spermatozoa, at least in hamsters (Suarez and Dai, 1992). Whether this property of the medium is really important for studying bovine sperm migration remains to be determined, since it is known that bull spermatozoa are not hyperactivated at ejaculation, although they still have to penetrate the cervical mucus at this point of time (Suarez and Dai, 1992). Despite the fact that frozen-thawed bull spermatozoa are prone to premature capacitation (Cormier et al., 1997), they do not hyperactivate as readily as hamster or mouse spermatozoa (Suarez and Dai, 1992; Chamberland et al., 2001), and so it might be difficult to find a relation between sperm migration and fertility in bulls, even if a more suitable medium such as polyacrylamide gel was used.

Second, in contrast to humans, in cattle the difference in sperm quality between bulls is a lot smaller and the number of individuals with very poor sperm quality is very low. For the evaluation of the sperm migration test in humans, spermatozoa of fertile men (donors of an AI program) were compared to spermatozoa of patients attending an infertility laboratory (Alexander, 1981; Insler et al., 1979), whereas in cattle the sperm migration test was performed with spermatozoa of bulls used for artificial insemination. These bulls have a high fertility resulting in a narrow range of NRRs, and therefore the number of individuals with poor sperm quality in cattle is low (Kummerfeld et al., 1981). In the only other comparable study of cattle a relation was found between fertility and sperm migration, but interestingly, in this study one bull with 50% NRR and one with 14% NRR were compared with 11 bulls with NRRs ranging from 60 to 70% (Murase et al., 1990). If the two bulls with the lowest NRRs were not included in the experiment, no relation could be found between NRR and the sperm migration distance. Even in the most carefully designed experiment in terms of examining the
same ejaculate for sperm migration as for NRRs, sperm migration would probably only discriminate between bulls with extremely varying NRRs.

Finally, the absence of a relation between sperm migration capacity and bull fertility could be explained by the fact that in dairy cattle, NRRs are determined after artificial insemination. During insemination the cervical barrier that spermatozoa have to overcome after natural mating is bypassed and the semen is deposited in the uterine body or in the uterine horns. It would be more logical to correlate sperm migration tests with fertility data obtained after natural mating, because then the spermatozoa have to migrate through the mucus before they reach the site of fertilization. It has been shown that sperm migration through cervical mucus in vitro could be indicative of the capacity of spermatozoa to populate the oviduct of goats (Cox et al., 1997). After semen deposition at the ostium uteri externum, spermatozoa from males with high migration efficiency populated the oviduct more effectively than that from males with low migration efficiency, but differences were not significant. Apart from these findings in vivo, no correlation was found between migration efficiency and in vitro fertilization rates (Cox et al., 1997). This may imply that capillary sperm migration tests may have value in predicting fertility under in vivo conditions, because the test is able to select superior sperm samples.

In conclusion, we have shown that sperm migration through MC, albeit a repeatable test, could not be linked with in vivo bull fertility. Sperm migration through a viscous medium is only a very limited aspect of sperm behaviour that has to be performed in preparation to fertilize the oocyte. To obtain more precise prediction of in vivo fertility it is imperative to combine different in vitro test systems as had been proposed by several authors (Zhang et al., 1999; Larsson and Rodriguez-Martinez, 2000).
REFERENCES


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CHAPTER 7

ASSESSMENT OF A NEW UTERO-TUBAL JUNCTION INSEMINATION DEVICE IN DAIRY CATTLE


Verberckmoes S., Van Soom A., Dewulf J., de Kruif A.
ABSTRACT

A new artificial insemination device for semen deposition near the utero-tubal junction in cattle (Ghent device) has been developed at the University of Ghent (Belgium). In this study, the effect of the new insemination device on sperm quality was evaluated. Moreover, in a field trial 4064 dairy cows were inseminated by 12 inseminators to examine the efficacy of the device under field conditions.

The Ghent device is a disposable plastic catheter which can easily follow the curvature of the uterine horns and thus reach the utero-tubal junction (UTJ). After expulsion of the inseminate with 0.7 ml or 1.7 ml of air, 19.0% of the insemination dose remained in the insemination catheter. Sperm loss can be diminished to 9.0% of the original insemination dose when the insemination catheter is flushed with 0.1 ml of air, followed by 0.6 ml of physiological saline solution. No toxic effect of the insemination catheter on sperm quality or fertilizing capacity was found. In the field trial, sperm were inseminated in dairy cattle which were divided in 3 groups. The first group was inseminated in the uterine body with the conventional insemination device, the second group in the uterine body with the Ghent device, and the third group in the tip of both uterine horns with the Ghent device. Each insemination was performed with 10-15 x 10^6 frozen-thawed spermatozoa. The pregnancy rates (PRs) were significantly affected by the insemination technique (P = 0.02), by the inseminator (P = 0.01), by heifer or cow (P < 0.01), and by the insemination number (P < 0.01). Pregnancy rates obtained with the conventional insemination device (57.6%) were significantly better than those obtained with the Ghent device in the uterine body (52.7%) (P < 0.01), but did not differ significantly from those obtained after deep insemination into both uterine horns (53.8%) (P = 0.27). It can be concluded that the Ghent device is suitable for utero-tubal junction insemination of dairy cattle under field conditions. Whether the Ghent device is also suitable for insemination with lower insemination doses is at present under investigation.
INTRODUCTION

Currently most commercial artificial inseminations (AI) in cattle are performed into the uterine body by means of a stainless steel insemination device of Cassou (Noakes et al., 2001). This rigid device facilitates the passage of the cervix and is covered with a plastic sheath to minimize transmission of infectious agents by the insemination device. Deposition of the inseminate into the uterine body of the cow makes it possible to achieve acceptable pregnancy rates (PRs) with a relatively low dose of frozen-thawed spermatozoa (10-15 million) (Uwland, 1984, Smidt and Zuidberg, 1988; Foote and Kaproth, 1997; Den Daas et al., 1998).

Lowering the insemination dose without a concomitant reduction in pregnancy rates can be obtained when fresh diluted semen is used (Shannon and Vishwanath, 1995). However, using fresh semen complicates distribution logistics and enhances the risk of spreading of infectious agents, e.g. foot-and-mouth disease virus. Deposition of semen closer to the oviduct could be an alternative to obtain acceptable pregnancy rates with lower insemination doses, less fertile semen, such as sex-separated spermatozoa or spermatozoa whose DNA-status may have been modified by specific treatment (Gandolfi, 2000; Hunter and Greve, 1998). Semen deposition near the utero-tubal junction will result in a decreased loss of spermatozoa by 1) retrograde flow in the cervical mucus (Larsson and Larsson, 1985; Mitchell et al., 1985; Nelson et al., 1987) and 2) phagocytosis during migration through the uterus (Hawk, 1983), and in an increased survival time of spermatozoa in the sperm-friendly environment of the isthmus (Suarez, 2001).

A good deal of research, with varying success, has been performed to improve the pregnancy rates (PRs) in cattle by deposition of spermatozoa closer to the place of fertilization. In most cases, no difference was found between deep uterine insemination (in uterine horns) and insemination in the uterine body (Knight et al., 1951; Stewart and Melrose, 1952; Olds et al., 1953; Macpherson, 1968; Hawk and Tanabe, 1986; Williams et al., 1988; McKenna et al., 1990) In one study, worse PRs were found after deep uterine insemination (Marshall et al., 1989) while four other research groups obtained better results after deep uterine insemination (Zavos et al., 1985; López-Gatius et al., 1988, Senger et al., 1988; Dalton et al., 1999). An explanation for the varying results can be found in the different insemination doses used, the application of uni- or bilateral insemination, the skill and
experience of inseminators, and the insemination of nulli- and pluriparous cows in natural heat or after estrus induction. The large variation in experimental set up and the fact that “deep uterine insemination” is not consistently defined, makes it difficult to compare the results of these different studies.

However, semen deposition near the utero-tubal junction can not be performed properly with the conventional rigid insemination device, since the contractility of the uterus in estrous cows is increased, and the uterine horns are curled (Noakes et al., 2001). Introduction of a rigid insemination device up to the tip of the uterine horns will enhance the risk of damaging the friable uterine wall with reduced fertility as a result (Wolff et al., 1990). Substituting the conventional insemination device for a new instrument which would be useful for UTJ-insemination meant that a device, which was both rigid enough to penetrate the cervix and flexible enough to follow the curves of the curled uterus, had to be developed. Moreover, the device had preferably to be made of disposable materials for sanitary reasons, had to be cheap to produce, and had to be non-toxic for spermatozoa.

In this article the characteristics of the catheter as well as the effect of the device on sperm quality and fertilizing ability were investigated. Moreover, a field trial was undertaken to compare PRs obtained after bilateral utero-tubal junction (UTJ) insemination and those obtained after insemination with the conventional insemination device. In both cases inseminations were performed with frozen-thawed semen in dairy cows and heifers in natural heat. A second goal of this field trial was to evaluate the feasibility of the newly developed Ghent device under field conditions.

MATERIAL AND METHODS

Semen

Frozen-thawed semen of Holstein Friesian bulls (n = 290) was generously supplied by the AI-centre, Holland Genetics (The Netherlands). No selection was made in bulls used for the field trial. The semen was extended in a Tris-egg yolk diluter, packaged in 0.25 ml French straws (Instruments de Médecine Vétérinaire, l’Aigle, France) frozen and stored in liquid nitrogen until use. Each straw contained 10-15 x 10^6 frozen spermatozoa. Before insemination, semen was thawed for 1 min in 37°C water. For the conventional insemination method, straws were opened at one end and introduced in the insemination device. For the
new insemination device, thawed semen was suspended in a vial containing 0.25 ml of 120 mM Na-citrate and subsequently aspirated into a flexible catheter that was introduced in the Ghent device.

Selection of cows for the insemination trial

Both dairy and dual purpose cows from different dairy farms (n = 1063) were selected for insemination, if the cows were in good general condition and seen in estrus by the farmer. Exclusion criteria were: 1) complicated previous parturition, and 2) abnormal, malodorous vaginal discharge. For data processing a difference was made between nulliparous heifers and cows.

Inseminators

Inseminations were performed by 12 veterinarians of the Flemish AI-centre (VRV). When the field trial was finished, the inseminators were asked to fill in a questionnaire to evaluate the suitability of the Ghent device under field conditions.

Estrus detection and AI

Cows (n = 3428) and nulliparous heifers (n = 636) in natural heat were artificially inseminated. Estrus detection was performed by the farmer. Animals with behavioral estrus in the morning were inseminated in the evening, while animals showing signs of standing estrus in the evening were inseminated the next morning. The conventional and new insemination techniques were performed under rectal guidance. Before introduction of the Ghent device into the female genital tract, the inseminator had to 1) thaw the straw, 2) suspend the semen (0.25 ml) into a vial with 0.25 ml of 120 mM Na-citrate, 3) aspirate the contents into the catheter by means of a syringe which was connected to the end of the catheter, and finally 4) introduce and fix the catheter filled with semen in the Ghent device. The syringe used to fill the catheter remained connected to the catheter and was used to expel the semen. Then the Ghent device was manipulated through the cervix and when the tip of the insemination device had entered the uterine body, the inner tube of the device was pushed forward and half of the insemination dose was deposited in the tip of the left uterine horn. Then the inner tube was pulled back into the uterine body, and subsequently pushed forward into the tip of the right uterine horn, where the second half of the insemination dose was deposited. Before the onset of the field trial each inseminator received 100 Ghent devices to practice.
**Characteristics of the Ghent device**

The Ghent device consists of 2 hollow plastic tubes, in which a catheter filled with semen is introduced (Verberckmoes et al., 2002). The outer tube is completely rigid, while the inner tube consists of a rigid caudal end and a flexible cranial tip. The outer plastic tube can move independently from the inner tube (Figure 1).

![Ghent device with a telescopic, flexible tip.](image)

The dimensions of total device, outer tube, rigid part of the inner tube, flexible part of the inner tube and the catheter that contains the inseminate are determined in mm (Table 1). The catheter consists of polyethylene, and the tubes are made of ABS and PVC. The Ghent Device and the new insemination technique are protected by a patent (01976188.1-2318-EP011181, Ghent University).

<table>
<thead>
<tr>
<th></th>
<th>Length (mm)</th>
<th>Internal diameter (mm)</th>
<th>External diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Outer tube</td>
<td>496.7</td>
<td>3.4</td>
<td>6.2</td>
</tr>
<tr>
<td>2a. Inner tube, rigid part</td>
<td>476.1</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>2b. Inner tube, flexible part</td>
<td>223.0</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>3. Insemination catheter</td>
<td>709.8</td>
<td>1.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Experiment 1: Determination of the percentage of spermatozoa which remains in the insemination catheter after insemination.**

To determine the number of frozen-thawed spermatozoa which remain in the insemination catheter after expelling the inseminate, the weight of 10 catheters was determined at different moments. When the semen was expelled using only air, the weight of the catheter was assessed 1) before filling, 2) when it was filled with diluted semen, 3) after expelling the semen with 0.7 ml of air and 4) when the catheter was flushed with an additional 1.0 ml of air. First the insemination catheter was filled with 0.5 ml of diluted sperm by means of aspiration with a syringe. The specific gravity of the Tris-egg yolk diluter in which the semen was
frozen is 1.03 g/ml. The concentration of the spermatozoa in the diluter, determined by means of a Bürker counting chamber, was 48 x 10^6 spermatozoa/ml. Since we determined how much semen remained in the catheter (in g), and since we knew the specific gravity of the sperm diluter, we were able to calculate the remaining volume of diluted semen in the catheter: weight (g) / specific gravity (g/ml) = volume (ml). The remaining volume of diluted semen multiplied by sperm concentration gives the exact number of spermatozoa that remained attached to the wall of the insemination catheter: volume (ml) x concentration (number/ml) = exact number. The remaining number of spermatozoa can be expressed as a percentage of the total insemination dose: number of spermatozoa remaining in the insemination catheter divided by the number of spermatozoa in insemination dose = % of insemination dose remaining in the insemination catheter.

When the semen was expelled with 0.1 ml air followed by 0.6 ml of physiological saline solution, the weight of 10 catheters was determined 1) before filling, 2) when filled with diluted semen, and 3) after expelling the diluted semen with 0.1 ml of air and 0.6 ml of physiological saline solution. When the insemination catheter was flushed with 0.1 ml of air and 0.6 ml of physiological saline solution, 0.56 ml of the flushing medium remained in the catheter (content of the insemination catheter). Subsequently, the content of the insemination catheter was expelled by air pressure and the sperm concentration in this medium was determined by means of a Bürker counting chamber. The remaining volume of physiological saline solution in the catheter multiplied by the sperm concentration gives the number of spermatozoa that remained attached to the wall of the insemination catheter and can be expressed as a percentage of the total insemination dose, agreeing with the previously described formula.

**Experiment 2: Effect of the insemination catheter on sperm quality.**

Sperm treatment: Fresh semen of 3 bulls was collected by means of an artificial vagina. The sperm concentration was determined by means of a Bürker counting chamber and light microscopy. The spermatozoa were diluted to a concentration of 30 x 10^6 spermazoa/ml in physiological saline solution. For each of the 3 bulls, the diluted spermatozoa were incubated for 1h at 22°C in: 1) 5 insemination catheters and, as a control, 2) in 1 non toxic cryotube (Nunc®, Wiesbaden, Germany). The sperm quality was determined before and after 1h incubation in the insemination catheters and cryotubes.
Sperm quality evaluation: The assessed sperm quality parameters were membrane integrity (MI) and motility and acrosome integrity (AI). The membrane integrity was determined by means of LIVE/DEAD® Sperm Viability Kit (Molecular Probes) and fluorescent microscopy. The sperm motility was determined with the Hamilton Thorne (HTM-Ceros 12.1) on at least 500 spermatozoa, and the following parameters were assessed: velocity average pathway (VAP), velocity straight line (VSL), velocity curved line (VCL), straightness (STR), linearity (LIN), percentage of progressive motile spermatozoa (PMS) and rapid progressively motile spermatozoa (RMS). The acrosome integrity (AI) was assessed by means of Pisum Sativum Agglutinin (PSA) staining with a Leica DMR fluorescence microscope (x 400) (Kawakami et al., 1993). Spermatozoa with fluorescent green heads indicated acrosome intact spermatozoa. Spermatozoa were considered as acrosome-reacted when a fluorescent band was observed at the equatorial region.

Experiment 3: Field trial.

In a field trial, the effect of insemination technique on PRs was investigated. The inseminations were performed from the 20th of September until the 1st of November 2001. Animals were divided at random into 3 groups. In the first group (n = 1416), animals were inseminated with the conventional insemination device in the uterine body. In the second group (n = 1334), semen was deposited in the uterine body with the Ghent device. In the third group (n = 1314), semen was deposited in the tip of both uterine horns with the Ghent device. Randomisation was performed by computer and the animals included in the field trial at a given day, were all inseminated with the same insemination technique.

At the moment of insemination the following data were registered by means of a hand terminal: identity of the cow, identity of the bull used, number of insemination (1st, 2nd, ≥3rd AI), heifer or cow, identity of the farmer, date of insemination, insemination technique used and identity of the inseminator. The 56 days non-return rate (NRR) was the percentage of animals that were not presented for AI during the 56 days after the first AI, and was based on the data obtained with the hand terminal. The effective pregnancy rates (PRs) were determined at 45-90 days after last insemination by the inseminators by means of rectal examination of the animals that were not offered again for AI. During the field trial, the inseminators were asked to fill in a questionnaire concerning the handiness of the Ghent device and the time spent per insemination.
STATISTICAL ANALYSIS

The differences in remaining sperm numbers between different flushing methods, were compared by means of one-way ANOVA and Scheffé post-hoc tests (SPSS, 10.0 Chicago, USA). Different sperm quality parameters were compared using a linear mixed effect model with an autoregressive correlation structure of the first order (S-Plus, 2000; Cambridge, USA). For the results obtained in the field trial, the effect of insemination technique, inseminator, parity of the cow, insemination number and week of insemination on the PRs was analysed by means of univariate and multivariate logistic regression. In the multivariate analysis, all significant parameters of the univariate analysis (P < 0.05) were included, and all 2-way interactions were tested (SPSS, 10.0, Chicago, USA).

RESULTS

Characteristics of the Ghent device.

The dimensions of the length, the internal and external diameter of the outer tube, the rigid part of the inner tube, the flexible part of the inner tube and of the catheter that contains the inseminate are given in Table 1. A small section of the flexible telescopic part of the inner tube (197.4 mm) protrudes from the outer tube. The content of the insemination catheter is 0.56 ml ($\pi r^2 L$). The Luer Lock system at the back of the catheter enables the connection of a disposable syringe.

Experiment 1: Determination of the number of spermatozoa which remain in the insemination catheter after insemination.

Although there was a displacement of 0.5 ml of air in the syringe by moving the plunger backwards, the catheter was loaded with only 0.46 g ± 0.05 g of diluted sperm. Extra flushing of the catheter with 1 ml of air had no significant effect (P = 0.37) on the remaining number of spermatozoa in the catheter (Table 2). The remaining number of spermatozoa was significantly decreased by expulsion of the inseminate with 0.1 ml of air followed by 0.6 ml of physiological saline solution (P < 0.01) (Table 2).
Table 2: Remaining spermatozoa (mean ± S.D.) after flushing the insemination catheter with 0.7 ml of air. 0.7 + 1.0 ml of air and 0.1 ml of air + 0.6 ml of physiological saline solution.

<table>
<thead>
<tr>
<th>Number of measurements</th>
<th>Remaining spermatozoa</th>
<th>% of insemination dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>Millions</td>
</tr>
<tr>
<td>0.7 ml air</td>
<td>n = 10</td>
<td>4.28 ± 0.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.7 + 1.0 ml air</td>
<td>n = 10</td>
<td>3.96 ± 0.85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 ml air + 0.6 ml PS</td>
<td>n = 10</td>
<td>2.18 ± 0.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>ab</sup> Different superscripts indicate a significant difference (P < 0.01). PS: physiological saline

**Experiment 2: Effect of the insemination catheter on sperm quality.**

Of all parameters determined, no significant differences could be found between the spermatozoa incubated in the insemination catheter and those incubated in the cryotubes (P > 0.05) (Table 3). After 1 h of incubation at 22°C, all sperm motility parameters were decreased with the exception of velocity curved line (VCL) and amplitude lateral head displacement (ALH). The small increase in VCL and ALH and decrease in the other sperm motility parameters indicate an insignificant deterioration of sperm quality during the 1h incubation period in both containers. For spermatozoa incubated in cryotubes and insemination catheters, the percentage of membrane-intact spermatozoa decreased insignificantly (P = 0.49) from 66.8% ± 8.8% at T0, to 50.3% ± 11.4% and 41.6% ± 12.1% respectively. The acrosome integrity (AI) remained unchanged (P = 0.88) at both times, in both containers (97%).

Table 3: Motility parameters (VAP, VSL, VCL, BCF, STR, LIN, PMS, RMS) analysed by means of a Hamilton Thorne analyzer of fresh semen immediately after collection (T0) and after 1h incubation (T1) at 22°C in cryotubes (control) and in the insemination catheters (mean ± S.D.).

<table>
<thead>
<tr>
<th>VAP (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VCL (µm/s)</th>
<th>STR (%)</th>
<th>LIN (%)</th>
<th>PMS (%)</th>
<th>RMS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>134.8 ± 11.2</td>
<td>113.0 ± 7.3</td>
<td>205.3 ± 32.5</td>
<td>82.9 ± 2.4</td>
<td>57.6 ± 5.4</td>
<td>51.4 ± 11.6</td>
</tr>
<tr>
<td>T1 control</td>
<td>120.7 ± 19.2</td>
<td>84.5 ± 4.5</td>
<td>220.0 ± 46.0</td>
<td>71.0 ± 8.7</td>
<td>41.0 ± 7.0</td>
<td>30.3 ± 13.6</td>
</tr>
<tr>
<td>T1 catheter</td>
<td>122.8 ± 6.2</td>
<td>91.6 ± 2.3</td>
<td>220.9 ± 25.3</td>
<td>75.4 ± 4.4</td>
<td>44.3 ± 6.2</td>
<td>39.4 ± 12.1</td>
</tr>
<tr>
<td>P-value</td>
<td>0.98</td>
<td>0.25</td>
<td>0.92</td>
<td>0.79</td>
<td>0.45</td>
<td>0.84</td>
</tr>
</tbody>
</table>

**Experiment 3: Field trial.**

A total of 3428 cows and 636 heifers were inseminated. Rectal examination to confirm pregnancy could be performed in 3902 animals (96%). Four per cent of the animals could not be examined, mostly because of culling before examination. As can be deduced from Table 4 the effective PRs were 15% lower than the NRRs.
Table 4: Non return rate (NRR) and pregnancy results after insemination with the conventional insemination device into the uterine body (CD body), with the Ghent device into the uterine body (GD body) and with the Ghent device into both uterine horns (GD horns).

<table>
<thead>
<tr>
<th>AI method</th>
<th>No. of AIs</th>
<th>NRR</th>
<th>Pregnant</th>
<th>Not pregnant</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD body</td>
<td>1416</td>
<td>72.0%</td>
<td>57.6%(^{a,b})</td>
<td>37.2%</td>
<td>5.2%</td>
</tr>
<tr>
<td>GD body</td>
<td>1334</td>
<td>68.0%</td>
<td>52.7%(^a)</td>
<td>43.4%</td>
<td>3.9%</td>
</tr>
<tr>
<td>GD horns</td>
<td>1314</td>
<td>69.0%</td>
<td>53.8%(^b)</td>
<td>43.5%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Total</td>
<td>4064</td>
<td>70.0%</td>
<td>54.7%</td>
<td>41.3%</td>
<td>4.0%</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Values with a different superscript in the same column differ significantly (P < 0.05).

The insemination technique, the inseminator, the parity of the cow, and the insemination number all had a significant effect on the PRs, as shown by univariate logistic regression (Tables 5, 6).

Table 5: Number of inseminations per inseminator and obtained pregnancy rates with different insemination techniques.

<table>
<thead>
<tr>
<th>Inseminator</th>
<th>No. of inseminations</th>
<th>Pregnancy rates (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD body</td>
<td>GD body</td>
</tr>
<tr>
<td>1</td>
<td>298</td>
<td>51.6</td>
<td>46.2</td>
</tr>
<tr>
<td>2</td>
<td>375</td>
<td>69.7</td>
<td>51.9</td>
</tr>
<tr>
<td>3</td>
<td>359</td>
<td>50.7</td>
<td>46.9</td>
</tr>
<tr>
<td>4</td>
<td>517</td>
<td>57.9</td>
<td>59.5</td>
</tr>
<tr>
<td>5</td>
<td>371</td>
<td>58.2</td>
<td>48.4</td>
</tr>
<tr>
<td>6</td>
<td>302</td>
<td>64.7</td>
<td>56.3</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>61.5</td>
<td>40.6</td>
</tr>
<tr>
<td>8</td>
<td>242</td>
<td>56.0</td>
<td>42.5</td>
</tr>
<tr>
<td>9</td>
<td>313</td>
<td>58.9</td>
<td>56.0</td>
</tr>
<tr>
<td>10</td>
<td>364</td>
<td>65.9</td>
<td>61.4</td>
</tr>
<tr>
<td>11</td>
<td>393</td>
<td>54.1</td>
<td>50.0</td>
</tr>
<tr>
<td>12</td>
<td>312</td>
<td>44.8</td>
<td>57.0</td>
</tr>
</tbody>
</table>

CD = Cassou device, GD = Ghent device
Table 6: Results of univariate analysis, examining the effect of insemination technique, inseminator, parity of the cow, insemination number and week of insemination on PRs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insemination technique</td>
<td></td>
</tr>
<tr>
<td>CD body vs GD horns</td>
<td>0.03</td>
</tr>
<tr>
<td>CD body vs GD body</td>
<td>0.05</td>
</tr>
<tr>
<td>GD horns vs GD body</td>
<td>0.01</td>
</tr>
<tr>
<td>Inseminator</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Parity of the cow</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Insemination number</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>First 2 weeks vs last 2 weeks of insemination</td>
<td>0.07</td>
</tr>
</tbody>
</table>

CD body: insemination with the conventional insemination device into the uterine body
GD body: insemination with the Ghent device into the uterine body
GD horns: insemination with the Ghent device into both uterine horns

To examine the effect of gaining experience with the Ghent device, the PRs of each inseminator obtained after deep insemination during the first 2 weeks were compared with those obtained during the last 2 weeks of the field trial. No significant differences could be detected (P = 0.07) although there was a tendency that experience improved the results (Table 6). The results of the multivariate analysis are presented in Table 7. This model, which takes several parameters into account, confirmed that the insemination technique, the inseminator, the parity of the cow and the insemination number significantly affected the PRs. It also demonstrated that significantly better PRs were obtained with the Ghent device in the tip of both uterine horns than with the Ghent device in the uterine body (P < 0.01), whereas no significant difference was found between insemination with the Ghent device in the tip of both uterine horns, and the conventional insemination technique (P = 0.27) (Table 7). When PRs of heifers and cows were analysed separately, identical PRs were obtained in heifers inseminated in the tip of both uterine horns and heifers inseminated in the uterine body with the Ghent device (63.3%), while non-significantly higher PRs were obtained in cows after insemination with the Ghent device in the tip of both uterine horns (52.8%) than in the uterine body (50.6%). The higher the insemination number, the lower the PRs with each of the insemination techniques. The questionnaire demonstrated that bilateral deep insemination with the Ghent device took 6.5 ± 2.3 min more than the conventional insemination technique, and that in 7% of the UTJ inseminations, clear blood was present at the tip of the insemination device. However, in about 45% of the UTJ inseminations at least a trace of blood could be observed.
Table 7: Results of the multivariate analysis, examining the effect of insemination technique, inseminator, parity of the cow and insemination number on PRs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insemination technique</td>
<td>0.02</td>
</tr>
<tr>
<td>CD body v.s. GD horns</td>
<td>0.27</td>
</tr>
<tr>
<td>CD body v.s. GD body</td>
<td>0.07</td>
</tr>
<tr>
<td>GD horns v.s. GD body</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Inseminator</td>
<td>0.01</td>
</tr>
<tr>
<td>Inseminator x technique</td>
<td>0.06</td>
</tr>
<tr>
<td>Parity of the cow</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Insemination number</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

DISCUSSION

In this study, we describe the use of a new artificial insemination device for cattle, which unlike the conventional Cassou insemination device, can be used for deep uterine insemination near the utero-tubal junction. Results of the present study indicate that our new Ghent device permits utero-tubal junction insemination in cattle under field conditions with satisfactory pregnancy rates. In addition, only a limited proportion of the inseminate is retained within the catheter, and no negative effect of the device on sperm quality was observed. This makes the Ghent device a possible useful instrument for artificial insemination with sexed or low doses of semen. The application of the device for low dose insemination implicates that a minimal number of spermatozoa may be lost during the insemination procedure. We have shown that flushing of the insemination catheter with 0.1 ml of air followed by 0.6 ml of physiological saline solution resulted in a sperm loss which comprised less than 10% of the initial inseminate. Although further increase of the flushing volume could reduce the remaining number of spermatozoa in the insemination catheter, we do not recommend doing so. The higher volume of flushing medium would decrease the number of spermatozoa entering the oviduct as a result of the increase in sperm loss by retrograde flow. Therefore, insemination near the UTJ requires low volume insemination doses since it will increase the percentage of spermatozoa that reach the isthmus of the oviduct, where they are protected from phagocytosis in the hostile uterine environment (Morris and Allen, 2002). A higher insemination volume would decrease the number of spermatozoa that enters the oviduct due to the increased retrograde flow.
As a control for any possible toxic effect of the insemination catheter on the spermatozoa, we examined sperm motility parameters as assessed by means of computer assisted sperm analysis. It is known that cytotoxic components can alter the motility of the spermatozoa, which depends upon intact cellular structures and functions, and which consequently offers an endpoint for quantitative evaluation of the cytotoxic potential of chemical substances (Seibert et al, 1989). Spermatozoa were incubated in physiological saline solution which contains no substances that could act as scavengers for toxic substances. Media containing serum or chelating agents such as BSA or EDTA are known to overcome (partially) the detrimental effect of cytotoxic components (Van Langendonckt et al., 1996; Erbach et al., 1995; Borenfreund, 1986). Even under conditions as described in experiment 2, no differences in motility parameters (VAP, VSL, VCL, BCF, STR, LIN, PMS, RMS) could be observed for spermatozoa which had been in contact with the Ghent device for 1 h. Moreover, no effect on in vivo fertilizing capacity of the same spermatozoa could be detected (data not shown).

Since we know that the Ghent device is not toxic for spermatozoa, and does not result in important sperm losses, it was somewhat disappointing to obtain no significant difference (P = 0.27) in PR after utero-tubal junction insemination when compared with conventional insemination. However, using the multivariate analysis, which is the most correct representation of the real difference between insemination techniques since all influencing parameters are accounted for, significantly higher pregnancy rates were observed after insemination with the Ghent device in the uterine horns than in the uterine body (P < 0.01). Several explanations for the results obtained with the different insemination techniques are possible. The lower PRs obtained with the Ghent device than with the conventional insemination device can be explained by the increased number of manipulations that the semen had to undergo. After thawing, the semen is suspended in a vial with Na-citrate, aspirated into a catheter and subsequently introduced in the Ghent device. The extra manipulations are time consuming and may have a negative effect on the sperm quality. The fact that the worst results were obtained after insemination in the uterine body with the Ghent device, can be explained by the technical problems that occur with the Ghent device because it is not developed for insemination in the uterine body. The Ghent device is longer than the conventional insemination device, which complicates its fixation in the uterine body. Because the uterine body of the cow has a length of only 1-3 cm (Nickel et al., 1999) there is an increased risk of damaging the septum that separates both uterine horns when the insemination device is not appropriately fixed in the uterine body. Moreover, it was found that
after using the Ghent device, in some cases blood was sticking on the tip of the device. It is known that the endometrium is vulnerable at the moment of insemination, because of the hyperaemic condition of the endometrium during estrus (Dellman and Eurell, 1998). The blood and shreds of mucosa liberated into the uterine lumen may have a negative effect on the fertilizing capacity of the spermatozoa. As examined in Chapter 9, blood does not influence sperm quality, but can cause agglutination of the spermatozoa. Subsequently this agglutination may result in a reduced number of spermatozoa that can reach the site of fertilization, resulting in decreased fertilization rates. In humans, it is known that preventing endometrial damage during deep uterine insemination increases the chance of conception (Lavie et al., 1997). However, we also have to take into account that the deposition of spermatozoa closer to the site of fertilization, decreases the chance of contact with blood, and as a consequence, less chance for the spermatozoa to agglutinate.

Another factor that may have influenced the results is the fact that some experience with the technique was required to obtain better results. During the preliminary trial period, UTJ-inseminations were performed after an epidural anaesthesia. Few animals strained and the female genital tract was easy to manipulate. Despite this advantage, and the fact that an epidural anaesthesia does not affect sperm transport in the uterus (Thurmon et al., 1996) 11 of the 12 inseminators did not continue to use epidural anaesthesia after the preliminary trial period with the Ghent device. The main reason for this was saving time. Interestingly, one inseminator used epidural anaesthesia for all UTJ-inseminations and obtained 8% better PRs than with the conventional insemination technique. The importance of gaining experience with the Ghent device is also shown by the fact that, in the multivariate analysis, the interaction between inseminator and insemination technique was approaching significance (P = 0.06). This indicates that some inseminators obtain better results with the Ghent device than others. Although the inseminators had the impression that inseminations with the Ghent device were easier to perform when they gained more experience with the device, no significant difference was found between the PRs of the first 2 and the last 2 weeks of the field trial. However, increasing the number of inseminations per inseminator could result in a significant effect.

In conclusion, semen deposition near the utero-tubal junction in cattle by means of a minimally invasive technique can be performed with the newly developed Ghent device. The Ghent device is made of disposable materials, has no toxic effect on bovine semen, can easily be handled by one person, and is suitable under field conditions as proven in the field trial. A
beneficial effect of UTJ insemination with the Ghent device in cattle is expected when low insemination doses or less fertile semen will be used. The Ghent device could offer the solution for the application of sex sorted semen under field conditions.
REFERENCES


ASSESSMENT OF THE GHENT DEVICE FOR UTERO-TUBAL JUNCTION INSEMINATION IN DAIRY CATTLE WITH LOWER INSEMINATION DOSES
ABSTRACT

A new artificial insemination device for semen deposition near the utero-tubal junction (UTJ) in cattle (Ghent device) was developed at the University of Ghent (Belgium). In this study, UTJ insemination of dairy cows with the Ghent device was compared with the conventional insemination technique to evaluate the effect on pregnancy rates after insemination with different doses of semen.

Three field trials were performed in which the insemination dose was lowered to 8 million (trial 1), 4 million (trial 2), and finally to 2 million frozen-thawed spermatozoa (trial 3). In each field trial, cows were divided in 3 groups: the first group was inseminated with a full insemination dose \((12 \times 10^6)\) in the uterine body with the conventional insemination device, the second group with a lowered insemination dose in the uterine body with the conventional insemination device, and the third group with a lowered insemination dose in the tip of both uterine horns with the Ghent device. Over all experiments, pregnancy rates were significantly affected by inseminator \((p < 0.01)\), parity of the cow \((p < 0.01)\), bull \((p < 0.01)\) and breed of the bull \((p = 0.03)\). The fertility rates were not significantly affected by the insemination technique \((p = 0.15)\), number of insemination (first, second, or third) \((p = 0.22)\), or history of the bull \((p = 0.81)\). It can be concluded that decreasing the insemination dose from 12 to 2 million frozen thawed spermatozoa had no effect on pregnancy rate in our experiments, neither with the conventional insemination device, nor with the Ghent device.
INTRODUCTION

Due to the commercialization of sex-sorted semen, new interests have arisen recently for insemination of cattle with low doses and poorly fertile semen. The application of sex-sorted semen would increase the efficiency of milk and meat production (Seidel, 2003). Dairy cows with high genetic value could be used for the production of female offspring, while cows with lower value could be used for the production of male beef-crossbred calves with high meat quality and good growth performance. However, an insemination dose of commercially available sex-sorted semen contains on average 1 - 2 x 10^6 frozen-thawed spermatozoa, which is 10% of the conventional insemination dose (10 – 20 x 10^6 spermatozoa) in cattle. Moreover, the high speed selection procedure which is used for commercial semen sexing has a detrimental effect on the sperm quality (Schenk et al., 1999; Garner et al., 2001; Seidel et al., 2003).

The relation between number of spermatozoa inseminated and bull fertility was first proposed by Salisbury and Van Demark (1951). This relation was further explained by a model proposed by Schwartz et al. (1981), which relates the number of spermatozoa to the probability of conception, based on a Poisson distribution. The validity of the model was confirmed by a series of field trials (Den Daas, 1992, 1998). These field trials were performed with the Cassou insemination device in the uterine body with different doses of frozen-thawed semen, and have shown that bulls differ in their maximum non return rate (NRR) value and that this maximum is reached as the insemination dose is increased. The required insemination dose to obtain optimal NRRs was 15, 10 and 7.5 x 10^6 frozen-thawed spermatozoa for low, moderate and highly fertile bulls, respectively (Uwland, 1984). Apart from the insemination dose and bull fertility, other factors such as time of insemination, stage of estrus of the cow, parity of the cow, number of insemination after calving, and inseminator do affect NRRs (Macmillan and Curnow, 1977; Verberckmoes et al., 2004a).

A final factor which can affect the optimal insemination dose and related NNRs is the insemination technique. Most commercial artificial inseminations (AIs) in cattle are performed into the uterine body by means of the stainless steel Cassou device (Noakes et al., 2001). However, in horses (Morris and Allen, 2002) and pigs (Rath, 2003), semen deposition closer to the site of fertilization instead of in the uterine body has been shown to improve pregnancy rates (PRs) while using lower insemination doses and poorly fertile semen. In
cattle, a good deal of research has been performed to evaluate the effect of deep insemination. However, in most cases no difference was found between deep uterine insemination (in uterine horns) and insemination in the uterine body (Knight et al., 1951; Salisbury and Van Demark, 1951; Steward and Melrose, 1952; Olds et al., 1953; Macpherson, 1968; Hawk and Tanabe, 1986; Williams et al., 1988; McKenna et al., 1990). In one study, worse PRs were found after deep uterine insemination (Marshall et al., 1989) while five other research groups obtained better results after deep uterine insemination (Zavos et al., 1985; Lopez-Gatius et al., 1988; Senger et al., 1988; Dalton et al., 1999). The large variation in experimental set up and the fact that “deep uterine insemination” is not consistently defined, makes it difficult to compare the results of these different studies. Moreover, in none of these studies, has the effect of deep versus conventional insemination been investigated with low insemination doses or poorly fertile semen. A limited number of published trials have investigated the effect of low dose insemination (≤ 2 x 10^6 frozen-thawed spermatozoa) in lactating dairy cows has been investigated is limited (Den Daas et al., 1998; Andersson et al., 2004). A beneficial effect of deep insemination with low dose insemination and insemination with poorly fertile semen has been shown in both pigs and horses (Morris and Allen, 2002; Rath, 2003). A similar beneficial effect has been predicted for cattle (Hunter and Greve, 1998; Hunter, 2001; 2003), but this hypothesis has never been proven (Seidel et al., 1999).

In order to enable semen deposition near the UTJ without damaging the endometrium, a new insemination device (Ghent device) was developed (Verberckmoes et al., 2004a). The Ghent device is equipped with both a rigid part to penetrate the cervix and a flexible part to follow the curves of the curled uterus. Moreover, the device is made of non-toxic disposable materials to keep the price low and to prevent the spread of diseases by using the same insemination material for the insemination of different cows or at different farms (Verberckmoes et al., 2004a).

This study evaluated the effect of two different insemination techniques in combination with gradually decreasing doses of non-sexed semen on pregnancy rates. The insemination techniques applied were utero-tubal junction (UTJ) insemination with the Ghent device and conventional insemination in the uterine body with the Cassou device. In a first field trial, PRs obtained after bilateral UTJ insemination with 8 million of frozen-thawed spermatozoa were compared with PRs obtained after insemination with the same insemination dose with the Cassou device in the uterine body. In a second and a third field trial, the insemination dose was decreased to 4 and 2 million frozen-thawed spermatozoa, respectively. Each trial
included a control group of cows which were inseminated with the conventional insemination technique with 12 million frozen-thawed spermatozoa.

MATERIAL AND METHODS

Semen

Frozen-thawed semen of 11 Holstein Friesian bulls (5 Red and 6 Black) was generously supplied by the AI-centre, Holland Genetics (The Netherlands). Only semen from popular bulls with known NRRs (determined at 56 days post insemination) and which were still alive at the start of the experiment were used for the field trial. In a preliminary field trial the “sensitivity” of the different bulls for the decrease of the insemination dose was evaluated. Based on the results obtained in a preliminary field trial, bulls were divided into three groups (history of the bull). Group 1 consisted of bulls (n = 4) which were “sensitive” for AI with decreased insemination doses. The NRRs of these bulls decreased with 2.5 to 3.5% when the insemination dose was lowered from $20 \times 10^6$ to $5 \times 10^6$ spermatozoa. Group 2 consisted of bulls (n = 3) which were not sensitive for AI with decreased insemination doses, and group 3 consisted of bulls (n = 4) with unknown sensitivity for AI with decreased insemination doses.

The semen was collected by means of an artificial vagina, extended in a Tris-egg yolk diluent with a final glycerol concentration of 7% (w/v) and diluted to final concentrations of 24, 16, 8, and 4 million spermatozoa per mL. To determine the sperm concentration, 65 µl ejaculated semen was diluted into 9000 µl triton-isoton 0.2% solution, and from this suspension, again 65 µl was again diluted into 9000µl triton-isoton 0.2% solution by means of a Hamilton Microlab 500 (Omnilabo, Breda, The Netherlands). The concentration of this final dilution was assessed by means of a coulter counter (Beckman Coulter, Mijdrecht, The Netherlands) for each freezing operation and rechecked after thawing.

Subsequently, semen was packaged in 0.5 mL French straws (Instruments de Médecine Vétérinaire, l’Aigle, France), frozen and stored in liquid nitrogen until use. From one ejaculate of each bull, 4 groups of straws were produced containing 12, 8, 4, and $2 \times 10^6$ frozen spermatozoa per straw. Before insemination, semen was thawed for 1 min in 37°C water. For the conventional insemination method, straws were opened at one end and introduced into the insemination device. For the Ghent device, modifications in the filling and emptying system were performed after each field trial (see further).
Selection of cows for the insemination trial

Both dairy and dual purpose cows from different dairy farms were selected for insemination, if the cows were in good general condition and seen in estrus by the farmer. Exclusion criteria were: 1) complicated previous parturition, and 2) abnormal, malodorous vaginal discharge. Animals were divided at random into 3 groups. In the first group, animals were inseminated with the conventional insemination device in the uterine body with a full insemination dose (12 million). In the second group, animals were inseminated in the uterine body with the conventional insemination device with a lowered insemination dose (8, 4, and 2 x 10^6). In the third group, the lowered insemination dose (8, 4, and 2 x 10^6) was deposited in the tip of both uterine horns, half of the dose left and half of the dose right, with the Ghent device. Randomisation was performed by computer and the animals included in the field trial at a given day, were all inseminated with the same insemination technique.

Estrus detection and AI

Cows and nulliparous heifers in natural heat were artificially inseminated. Estrus detection was performed by the farmer. Animals with behavioural estrus in the morning were inseminated in the evening, while animals showing signs of standing estrus in the evening were inseminated the next morning. The conventional and new insemination techniques were performed under rectal guidance. Before introduction of the Ghent device into the female genital tract, the inseminator had to thaw the straw and fill the insemination catheter. Then the Ghent device was manipulated through the cervix and when the tip of the insemination device had entered the uterine body, the inner tube of the device was pushed forward and half of the insemination dose was deposited in the tip of the left uterine horn. Then the inner tube was pulled back into the uterine body, and subsequently pushed forward into the tip of the right uterine horn, where the second half of the insemination dose was deposited. The semen was expelled by means of a syringe which was connected on the back side of the Ghent device.

Data collection

At the moment of insemination the following data were registered by means of a hand terminal: used insemination technique and insemination dose, identity of the cow, identity of the bull, number of insemination (1st, 2nd, ≥3rd AI), nulliparous heifer or cow, identity of the farmer, date of insemination, insemination technique used and identity of the inseminator. The 56 day non-return rate (NRR) was the percentage of animals that were not presented for
AI during the 56 days after the first AI, and was based on the data obtained with the hand terminal. The effective pregnancy rates (PRs) were determined by the inseminators at 45-90 days after last insemination by means of rectal palpation of the animals that were not presented again for AI.

**Experimental design**

*Experiment 1: Pregnancy rates obtained with 8 million frozen-thawed spermatozoa inseminated with the conventional and the Ghent devices, compared to conventional insemination with 12 million frozen-thawed spermatozoa.*

In a first field trial, 269 heifers and 835 cows were inseminated by 8 inseminators. Three insemination methods were compared; group 1: insemination in the uterine body with the conventional insemination device with 12 million frozen-thawed spermatozoa, group 2: insemination in the uterine body with the conventional device with 8 million frozen-thawed spermatozoa, and group 3: insemination in the tip of both uterine horns with the Ghent device with 8 million frozen-thawed spermatozoa.

In this field trial, before insemination with the Ghent device, thawed semen was suspended in a vial and subsequently aspirated into a flexible catheter that was introduced in the Ghent device (Verberckmoes et al., 2004a).

*Experiment 2: Pregnancy rates obtained with 4 million frozen-thawed spermatozoa inseminated with the conventional and the Ghent devices, compared to conventional insemination with 12 million frozen-thawed spermatozoa.*

In the second field trial, 185 heifers and 683 cows were inseminated by 6 inseminators and a similar set up as in the first field trial was applied. The three insemination methods in this field trial were; group 1: insemination in the uterine body with the conventional insemination device with 12 million frozen-thawed spermatozoa, group 2: insemination in the uterine body with the conventional insemination device with 4 million frozen-thawed spermatozoa, and group 3: insemination in the tip of both uterine horns with the Ghent device with 4 million frozen-thawed spermatozoa.

In contrast to the first experiment, the insemination technique with the Ghent device was modified to increase the net number of spermatozoa expelled. Instead of emptying the insemination catheter with air, the catheter was flushed with 0.6 mL of physiological saline solution, reducing the sperm loss from 19% to 9% (Verberckmoes et al., 2004a).
Experiment 3: Pregnancy rates obtained with 2 million frozen-thawed spermatozoa inseminated with the conventional and the Ghent devices, compared to conventional insemination with 12 million frozen-thawed spermatozoa.

In the third field trial, 249 heifers and 384 cows were inseminated by 5 inseminators and a similar set up as in the previous field trials was applied. The three insemination methods in this field trial were; group 1: insemination in the uterine body with the conventional insemination device with 12 million frozen-thawed spermatozoa, group 2: insemination in the uterine body with the conventional insemination device with 2 million frozen-thawed spermatozoa, and group 3: insemination in the tip of both uterine horns with the Ghent device with 2 million frozen-thawed spermatozoa.

In this field trial, the filling system of the Ghent device was modified in such a way that Cassou straws could be emptied immediately into the insemination catheter without wasting semen (Figure 1). In the previous trials (trial 1 and 2), straws had to be thawed and the content had to be emptied into a small vial and subsequently aspirated into the insemination catheter. The latter handling was complicated, time consuming and enhanced the risk of sperm damage by cold shock.

Figure 1: Facilitation of the semen transfer of the Cassou straw into the Ghent device by means of a “straw emptier”; 1) inner tube of the Ghent device, 2) connection part of the Ghent device, 3) plastic tube containing Cassou straw, 4) plunger to empty the straw directly into the catheter of the Ghent device.

STATISTICAL ANALYSIS

First all parameters (insemination technique, inseminator, parity of the cow, insemination number, identity of the bull, breed of the bull, and history of the bull were analyzed by means of univariate logistic regression over all 3 experiments. Subsequently, parameters which were significant in the univariate, were also analysed by means of multivariate logistic regression. The group of cows inseminated with 12 million frozen-thawed spermatozoa was used as a
control group within each experiment. Since “bull” and breed of bull were highly correlated, only the breed of the bull was selected for the multivariate analysis.

RESULTS

The insemination doses of 12, 8, 4, and 2 million effectively consisted of $11.8 \pm 0.6 \times 10^6$, $8.1 \pm 0.5 \times 10^6$, $4.0 \pm 0.3 \times 10^6$, and $2.0 \pm 0.2 \times 10^6$ spermatozoa, respectively, as evaluated by Hamilton coulter counter.

Using the univariate logistic regression, no significant difference could be found between PRs obtained after AI with a full ($12 \times 10^6$) and a decreased insemination dose ($8, 4, \text{ and } 2 \times 10^6$) ($p = 0.15$) (Table 1).

Table 1: Non return rate (NRR) and pregnancy results after insemination with the conventional insemination device into the uterine body (CD body), with the Ghent device into the uterine body (GD body) and with the Ghent device into both uterine horns (GD horns).

<table>
<thead>
<tr>
<th>AI method</th>
<th>No. of AIs</th>
<th>NRR</th>
<th>Pregnant (%)</th>
<th>Not pregnant (%)</th>
<th>Unknown (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD, $12 \times 10^6$</td>
<td>390</td>
<td>70.8</td>
<td>49.5</td>
<td>42.3</td>
<td>8.2</td>
</tr>
<tr>
<td>CD, $8 \times 10^6$</td>
<td>342</td>
<td>69.2</td>
<td>54.1</td>
<td>41.2</td>
<td>4.7</td>
</tr>
<tr>
<td>GD, $8 \times 10^6$</td>
<td>428</td>
<td>68.4</td>
<td>48.4</td>
<td>47.4</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>1160</td>
<td>69.5</td>
<td>50.4</td>
<td>43.9</td>
<td>5.7</td>
</tr>
<tr>
<td>CD, $12 \times 10^6$</td>
<td>344</td>
<td>69.7</td>
<td>45.3</td>
<td>42.7</td>
<td>11.9</td>
</tr>
<tr>
<td>CD, $4 \times 10^6$</td>
<td>322</td>
<td>69.8</td>
<td>41.0</td>
<td>43.2</td>
<td>15.8</td>
</tr>
<tr>
<td>GD, $4 \times 10^6$</td>
<td>343</td>
<td>65.5</td>
<td>40.2</td>
<td>45.5</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>1009</td>
<td>68.4</td>
<td>42.2</td>
<td>43.8</td>
<td>14.0</td>
</tr>
<tr>
<td>CD, $12 \times 10^6$</td>
<td>225</td>
<td>69.8</td>
<td>51.1</td>
<td>44.0</td>
<td>4.9</td>
</tr>
<tr>
<td>CD, $2 \times 10^6$</td>
<td>220</td>
<td>69.5</td>
<td>51.8</td>
<td>40.0</td>
<td>8.2</td>
</tr>
<tr>
<td>GD, $2 \times 10^6$</td>
<td>238</td>
<td>69.5</td>
<td>47.9</td>
<td>43.3</td>
<td>8.8</td>
</tr>
<tr>
<td>Total</td>
<td>683</td>
<td>69.6</td>
<td>50.2</td>
<td>42.5</td>
<td>7.3</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Values with a different superscript in the same column differ significantly ($P < 0.05$).

Also no significant difference was found between the groups inseminated with decreased insemination doses either with the conventional or Ghent device (Table 2). PRs in the three
groups of the second experiment were lower than in experiment 1 and 3, albeit not significantly (p = 0.08). No significant difference was found between cows inseminated for the first (n = 2165), second (n = 637) or third or more time (n = 50) (p = 0.22). Univariate as well as multivariate analysis showed that PRs were significantly affected by inseminator, parity of the cow, bull, and breed of the bull (Table 2).

Table 2: Results of univariate analysis, examining the effect of insemination technique, inseminator, parity of the cow, insemination number, bull, breed of the bull and history of the bull on PRs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insemination technique</td>
<td>0.15</td>
</tr>
<tr>
<td>CD 12 x 10^6 vs GD 8 x 10^6</td>
<td>0.40</td>
</tr>
<tr>
<td>CD 12 x 10^6 vs GD 4 x 10^6</td>
<td>0.71</td>
</tr>
<tr>
<td>CD 12 x 10^6 vs GD 2 x 10^6</td>
<td>0.90</td>
</tr>
<tr>
<td>CD 12 x 10^6 vs CD 8 x 10^6</td>
<td>0.25</td>
</tr>
<tr>
<td>CD 12 x 10^6 vs CD 4 x 10^6</td>
<td>0.21</td>
</tr>
<tr>
<td>CD 12 x 10^6 vs CD 2 x 10^6</td>
<td>0.38</td>
</tr>
<tr>
<td>CD 8 x 10^6 vs GD 8 x 10^6</td>
<td>0.09</td>
</tr>
<tr>
<td>CD 4 x 10^6 vs GD 4 x 10^6</td>
<td>0.67</td>
</tr>
<tr>
<td>CD 2 x 10^6 vs GD 2 x 10^6</td>
<td>0.42</td>
</tr>
<tr>
<td>Inseminator</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Parity of the cow</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Insemination number</td>
<td>0.22</td>
</tr>
<tr>
<td>Bull</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Breed of bull</td>
<td>0.03</td>
</tr>
<tr>
<td>History of bull</td>
<td>0.81</td>
</tr>
</tbody>
</table>

CD body: insemination with the conventional insemination device into the uterine body
GD body: insemination with the Ghent device into the uterine body
GD horns: insemination with the Ghent device into both uterine horns
History of the bull = “sensitivity” for AI with decreased insemination doses in a preliminary field trial (sensitive, not sensitive, unknown).

The pregnancy rates obtained by the different inseminators varied from 37.8% to 55.5%. The average PR in heifers (n = 782) and cows (n = 2070) was 65.3% and 47.3% respectively (p < 0.01) (Figure 2).
UTJ insemination with lower insemination doses

Figure 2: Effect of insemination dose (12, 8, 4, and 2 x 10^6 spermatozoa) and insemination technique (conventional device (CD) versus Ghent device (GD)) on PRs in heifers and cows.

PRs obtained by Red Holstein bulls were 4.1% higher than those obtained by Black Holstein bulls (p < 0.01) (Table 3).

Table 3: Effect of breed of the bull on PRs.

<table>
<thead>
<tr>
<th>Breed of bull</th>
<th>No. of AIs</th>
<th>Pregnant (%)</th>
<th>Not pregnant (%)</th>
<th>Unknown (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black Holstein (n = 6)</td>
<td>1684</td>
<td>45.8</td>
<td>45.1</td>
<td>9.1</td>
</tr>
<tr>
<td>Red Holstein (n = 5)</td>
<td>1168</td>
<td>49.9</td>
<td>41.3</td>
<td>8.8</td>
</tr>
<tr>
<td>Total</td>
<td>2852</td>
<td>47.4</td>
<td>43.5</td>
<td>9.0</td>
</tr>
</tbody>
</table>

In the present study, no difference in PR was found between bulls which had shown to be sensitive (n = 4) or insensitive (n = 3) for AI with decreased insemination doses in a preliminary study (p = 0.81) (Table 4).

Table 4: PRs of bulls divides into three groups in accordance to their sensitivity for AI with decreased insemination doses in a preliminary field trial (history).

<table>
<thead>
<tr>
<th>History bull</th>
<th>No. of AIs</th>
<th>Pregnant (%)</th>
<th>Not pregnant (%)</th>
<th>Unknown (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not sensitive (n = 3)</td>
<td>857</td>
<td>47.5</td>
<td>42.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Sensitive (n = 4)</td>
<td>1072</td>
<td>48.0</td>
<td>44.1</td>
<td>7.8</td>
</tr>
<tr>
<td>Unknown (n = 4)</td>
<td>923</td>
<td>46.8</td>
<td>44.2</td>
<td>9.0</td>
</tr>
<tr>
<td>Total</td>
<td>2852</td>
<td>47.5</td>
<td>43.5</td>
<td>9.0</td>
</tr>
</tbody>
</table>
DISCUSSION

According to Hunter (Hunter and Greve, 1998; Hunter, 2001; 2003), deep uterine insemination would be a fruitful way forward to improve fertility rates in cattle with low doses and poorly fertile semen. However, in our study no beneficial effect of bilateral UTJ insemination with the Ghent device on the PRs could be observed, even when the insemination dose was decreased from 12 to 2 million frozen-thawed spermatozoa. Furthermore, no difference was found between semen depositions into the uterine body with the conventional device and bilateral UTJ insemination with the Ghent device.

In contrast to other studies in which the 56d NRR was used as a fertility parameter, the fertility assessment in our study was based on effective pregnancy rates (PR) determined by rectal palpation between 45 and 90 days after last AI. The effective PRs are more accurate and make it possible to evaluate the fertility based on a relatively low number of cows per group. In our study, the overestimation of the effective PR by NRR was about 21.0%. This is the result of not taking the natural breeding and culling after AI into account, which lead to higher NRRs. The NRR and the percentage of non pregnant cows differed only slightly between the 3 experiments (Table 1). The variation in PR was larger. This is probably the result of the relatively high number of unknown data (14.0%) in the second experiment.

Several large scale field trials using the conventional insemination technique with low insemination doses have been performed in the Netherlands (Uwland, 1984; Smidt and Zuidberg, 1988; Den Daas et al., 1998). These studies showed that with the conventional insemination technique a significant decrease in NRR could be obtained only when the inseminations were performed with less than $10 \times 10^6$ frozen-thawed spermatozoa. Therefore insemination doses of 8, 4 and $2 \times 10^6$ frozen-thawed spermatozoa were used in our study.

In the present study we did not only want to investigate the effect of low dose insemination on the PRs, but we also wanted to evaluate the hypothesis of Hunter, according to which deep intra-uterine insemination has a beneficial effect on the PRs due to the reduction of the sperm loss during migration through the uterus and the more efficient population of the functional sperm reservoir in the isthmus of the oviduct (Hunter and Greve, 1998; Hunter, 2001; 2003). That deep uterine insemination would favour the access of spermatozoa to the oocyte in cattle was shown by Dalton et al. (1999).
Although the effect of deep insemination has been studied extensively (Knight et al., 1951; Salisbury and VanDemark, 1951; Stewart and Melrose, 1952; Olds et al., 1953; Macpherson, 1968; Hawk and Tanabe, 1986; Williams et al., 1988; McKenna et al., 1990; Marshall et al., 1989; Zavos et al., 1985; López-Gatius et al., 1988; Senger et al., 1988; Dalton et al., 1999), nobody has been able to clearly demonstrate the beneficial effect of this technique. In most of these studies, moderate ($10 - 20 \times 10^6$) to high $\geq 20 \times 10^6$ doses of frozen-thawed semen were used. Moreover, in the studies performed in the early fifties relatively high numbers of fresh semen were used (Salisbury and VanDemark, 1951; Stewart and Melrose, 1952; Olds et al., 1953). It is not surprising that no significant difference was found between deep and conventional insemination in most of these studies, since the number of spermatozoa inseminated was not a limiting factor for fertilization. In our viewpoint, the beneficial effect of UTJ insemination compared to conventional AI, can only be demonstrated when the number of spermatozoa inseminated is the limiting factor for successful fertilization. This has very recently been investigated in a study by Andersson et al. (2004), in which the effect of low dose insemination ($2 \times 10^6$) in cattle in combination with deep and conventional insemination was examined. This study confirmed the hypothesis of Hunter since no difference was found between deep and conventional AI when $15 \times 10^6$ spermatozoa were used, while numerically, although not significantly ($p = 0.27$), higher PRs were obtained with $2 \times 10^6$ spermatozoa deposited into the horn ipsilateral to the side of the impending ovulation (Andersson et al., 2004). Similarly, in the present study we were not able to demonstrate a significant beneficial effect of deep insemination. This can be due to the fact that, in our study, the insemination doses used, were still not low enough to represent a limiting factor for fertilization. No significant difference in fertility was observed between AI with $12, 8, 4$ and $2 \times 10^6$ spermatozoa. Unexpectedly and in contrast to the other studies (Uwland, 1984; Smidt and Zuidberg, 1988; Den Daas et al., 1998; Andersson et al., 2004), the PRs did not decrease in our study. This finding was certainly not due to an incorrect insemination dose since each batch was checked before and after freezing and thawing. Sperm quality was also similar in all experiments since all insemination doses were produced from the same ejaculates and were processed and frozen in the same way and at the same moment. An important difference between our study and those performed by Uwland (1984), Smidt and Zuidberg (1988) and Den Daas et al. (1998), is however the fact that in our field trials inseminations were performed by well trained veterinarians with extensive experience in AI, whereas in the other studies inseminations were performed by technicians. In studies where conventional inseminations were performed by technicians, it has been shown that lack of
accuracy of semen deposition was an important reason for reduced fertility (Lopez-Gatius, 2000). In over 50% of such cases, inseminators were not sufficiently trained to deposit semen into the uterine body, so that intracervical insemination was often performed (Lopez-Gatius, 2000).

Another noticeable difference between our study and investigations by Andersson et al. (2004) is the fact that PRs did not decrease after deep insemination with $2 \times 10^6$ frozen-thawed spermatozoa. This could be due to the different site of semen deposition, and also to the insemination technique. In the study of Andersson et al. (2004) an embryo transfer pistolet with side openings (IMV blue sheets) was used and semen was deposited halfway one uterine horn and not bilaterally at the UTJ. Moreover semen deposition with rigid devices such as an embryo pistolet or a conventional insemination device near the UTJ cannot be performed properly in cows due to the curling of the uterine horns at the moment of insemination (Noakes et al., 2001). The use of such devices for deep insemination requires extra manipulations of the uterus and increases the risk of endometrial damage, which may cause a decrease in fertility (Andersson et al., 2004; Verberckmoes et al., 2004b). Apart from the fact that UTJ insemination may be more beneficial when low doses or poorly fertile semen is used, it is also more likely that this beneficial effect will be more obvious in multiparous cows which have much longer uterine horns than nulliparous heifers. Although in our study consistently higher PRs were obtained in nulliparous heifers than in multiparous cows, no beneficial effect of UTJ insemination was found in either heifers or cows. Here again, the insemination dose seemed to be no limiting factor for successful fertilization, not even in cows. In contrast to our previous field trial with the Ghent device (Verberckmoes et al., 2004a), the insemination number had no significant effect on the PRs. This can be explained by the fact that in the present study 98.3% of all inseminations were first and second inseminations after calving, while the difference in PR between first (48.4% ± 4.8%) and second inseminations (45.6% ± 4.8%) after calving was very small.

In the present study each half of the low insemination dose ($8, 4$ and $2 \times 10^6$) was deposited near the UTJ of each uterine horn with the Ghent device, and similar PRs were obtained as with the full insemination dose ($12 \times 10^6$) in the uterine body. However, since cows are generally single ovulators, only one oocyte per ovulation is released and descends into the left or right oviduct where fertilization occurs. Determination of the site of ovulation before insemination would enable semen deposition in the horn ipsilateral to the site of ovulation. This normally would result in a halving of the insemination dose without a
reduction in fertility. In other single ovulators such as horses and water buffaloes, deposition of low doses of frozen-thawed semen \((1 - 3 \times 10^6)\) near the UTJ ipsilateral to the site of ovulation has been shown to be effective (Morris et al., 2000a, 2000b; 2003; Presicce et al., 2004). However, in contrast to the latter studies in horses and water buffaloes, determination of the exact moment of insemination in cows is less accurate under field conditions. Moreover, since no ultrasound examination nor palpation of the ovaries is performed in cattle at the moment of insemination, the site of ovulation is unknown. Palpation of the ovaries in cattle is not done in order to avoid rupture of mature follicles. A beneficial effect of UTJ has also been demonstrated in pigs when low doses of poor fertile semen were used (Martinez et al. 2001; Grossfeld et al. 2003; Rath et al., 2003). However, since pigs are multiple ovulators, half of the insemination dose has to be deposited at each of both UTJs.

As in most field trials, a significant difference in PRs was obtained between inseminators \((p<0.01)\) and between different bulls \((p<0.01)\). Some bulls are more fertile than others as has been clearly shown by Den Daas et al. (1998). This research group demonstrated that the optimal insemination dose to obtain 95% of the maximal conception rate ranges from \(1 \times 10^6\) to \(11 \times 10^6\) frozen-thawed spermatozoa for conventional AI (Den Daas et al., 1998). The difference in fertility between bulls can only be demonstrated when suboptimal insemination doses are used. In most studies, no decrease in fertility can be observed when cows are inseminated with \(7.5 \times 10^6\) or more frozen-thawed spermatozoa (Smidt and Zuidberg, 1988; Uwland, 1984). Even when the bulls are divided into 3 groups in accordance with their NRR (low, moderate or highly fertile) no significant decrease in fertility can be observed when the insemination dose is decreased from \(20 \times 10^6\) \((n = 5426)\) to \(7.5 \times 10^6\) spermatozoa \((n = 5165)\) (Smidt and Zuidberg, 1988). However, in contrast to our study, Uwland (1984) did find a significant decrease in fertility for moderately and low fertile bulls when the insemination dose was decreased to 10 and \(7.5 \times 10^6\) frozen-thawed spermatozoa, respectively, but not for highly fertile bulls, not even when the insemination dose was reduced to \(3.75 \times 10^6\). Despite the fact that our study included not only highly fertile bulls, but also bulls of unknown fertility \((n = 4)\) and bulls which had shown to be sensitive for AI with decreased insemination doses \((n = 4)\), no difference could be demonstrated between the groups of bulls which were suspected to have a different sensitivity for AI with low insemination doses.

The higher PRs obtained with semen from Red rather than from Black Holstein Friesian bulls \((p = 0.03)\) is most likely due to a better semen quality, but can also be the result of the
higher fertility of the cows inseminated with this semen. However, based on our field trial, it is impossible to determine which one of both factors is the most important.

In conclusion, no decrease in fertility was observed when the insemination dose was decreased from 12 to 2 million frozen-thawed spermatozoa, neither with the Ghent device nor with the conventional insemination device. Further trials with even lower insemination doses are required to determine whether UTJ insemination with the Ghent device would be better than conventional insemination in cattle.
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CHAPTER 9

EFFECT OF WHOLE BLOOD AND SERUM ON BOVINE SPERM QUALITY AND IN VITRO FERTILIZATION CAPACITY


Verberckmoes S., Van Soom A., De Pauw I., Dewulf J., Rijselaere T., de Kruif A.
ABSTRACT

Under physiologic conditions, low concentrations of blood may be present in the uterine fluid of the estrous cow at the moment of insemination. To decrease the insemination dose and to obtain good insemination results with less fertile semen, more invasive insemination methods such as utero tubal Junction insemination can be used. However, more invasive insemination methods increase the risk of damaging the hyperaemic endometrium, with blood in the uterine fluid as result. In this study, the effect of 0%, 0.15% and 1.5% whole blood and serum on bovine sperm quality and in vitro fertilizing capacity was evaluated. Sperm quality as assessed by total motility, progressive motility, membrane integrity and acrosomal status was not affected by the presence of blood or serum (P > 0.05). However, the in vitro fertilizing capacity decreased with increasing concentrations of blood and serum (P < 0.01). The rate of polyspermy increased with increasing concentrations of serum (P < 0.01), but not with increasing concentrations of blood (P = 0.30). In conclusion, no immediate effect of blood and serum was visible on several sperm quality parameters, except for an increased prevalence of head to head agglutination (HHA). However, blood and serum did have a negative effect on in vitro fertilizing capacity.
INTRODUCTION

In practice, cows are inseminated about 12 h after the onset of behavioral estrus. Cows observed in estrus in the morning are inseminated the evening of the same day, while cows observed in estrus in the evening are inseminated the next morning (a.m. / p.m. schedule) (Noakes et al., 2001a). The standard technique of insemination is to grasp the cervix through the rectum with one hand. An insemination pipette, containing a semen straw is then passed through the vagina and manipulated into and through the cervix. The semen is then expelled into the small uterine body (Noakes et al., 2001a). In these circumstances, presence of blood on the insemination pipette is very rarely observed.

Utero tubal junction (UTJ) insemination is an alternative to standard inseminations with possible applications for sexed semen or less fertile semen. A new insemination device has been developed at our departement and has been used in a field trial (Verberckmoes et al., 2004). However when inseminations are performed deep in one or both uterine horns, traces of blood may be present on the tip of the insemination catheter in 45% of inseminations (Verberckmoes et al., 2002b). It is known that during estrus, the epithelium of the uterus is very hyperaemic, resulting in capillary haemorrhage and post-estrual bleeding (24-48 hrs post estrus) (Noakes et al., 2001b). Due to this hyperaemic state of the endometrium, an insemination device that is introduced deep into the uterine horns may very easily induce limited bleeding, without causing an inflammatory reaction of the endometrium. Nevertheless, the presence of blood at the time of insemination may interfere with sperm quality and subsequent fertilizing ability. Since it is very difficult to investigate the effect of blood upon fertilization under in vivo conditions, it was the purpose of this study to evaluate the effect of whole blood and serum on bovine sperm quality and fertilizing capacity in vitro. Concentrations of blood and serum were chosen in accordance to the concentrations detected in the uterine fluid of estrous cows (Anderson et al., 1985). Anderson et al. (1985) found 10 million erythrocytes per ml uterine fluid, which equals a 0.15% of whole blood (v:v ratio). The fertilizing capacity of the spermatozoa was investigated by means of routine in vitro fertilization in the presence of 0, 0.15 and 1.5% blood and serum.
MATERIALS AND METHODS

Media.

To evaluate the effect of whole blood and serum on sperm quality, spermatozoa were incubated in isotonic Hepes-TALP medium. This medium contains 10 mM Hepes, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 2 mM sodium bicarbonate, 10 µg/ml gentamycin sulphate and 3 mg/ml bovine serum albumin (BSA). Oocytes used for IVF experiments were incubated in maturation medium consisting of modified bicarbonate buffered TCM199 medium (Gibco BRL, Merelbeke, Belgium) supplemented with 20% heat-inactivated Foetal Calf Serum (FCS) (N.V. Hyclone Europe S.A., Erembodegem, Belgium), 0.2 mM sodium pyruvate (Sigma-Aldrich, Bornem, Belgium), 50µg/ml gentamycin sulphate (Gibco BRL, Merelbeke, Belgium) and 0.4 mM glutamine (Sigma-Aldrich, Bornem, Belgium).

Collection of blood and serum.

Blood was taken from an estrous cow by means of a vacutainer containing 0.125 M Na Citrate (BD vacutainer systems, Plymouth, UK), resulting in negligible citrate concentration in the blood cultures. The concentration of red blood cells (RBC) was determined by means of a Bürker counting chamber after a dilution of $10^3$ in Hepes TALP. One ml of whole blood contained $6 \times 10^9$ red blood cells (RBC). Another blood sample of the same cow was taken by means of a venoject® (Terumo Europe NV, Leuven, Belgium) for the production of bovine serum. After 5h of incubation at room temperature, the clotting process was terminated, and the serum was subsequently separated and stored at 5°C.

Sperm treatment.

For all experiments, frozen-thawed semen from the same bull ejaculate was separated on a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient (90% and 45% Percoll solutions in a Hepes buffered salt solution). The concentration of the semen sample was determined by means of a Bürker counting chamber. For the evaluation of the effect of whole blood and serum on the sperm quality, spermatozoa were diluted in Hepes TALP containing 0, 0.15 and 1.5% of whole blood or serum. Sperm quality was assessed after 30 min and again after 5 hrs of incubation at 38.5°C. These time periods were chosen because normally the first spermatozoa reach the isthmus within 30 min after AI and fertilization occurs a few hours
Effect of whole blood and serum on bovine sperm quality and in vitro fertilizing capacity later (Hawk, 1983). For the evaluation of the effect of whole blood and serum on the IVF capacity, spermatozoa were diluted in IVF-TALP with 0, 0.15 and 1.5% of whole blood and the same concentrations of serum.

**Oocyte maturation and fertilization.**

Cow ovaries were randomly selected in a slaughterhouse and transported to the laboratory within 3 to 4 hours after collection. Follicles of 2 to 6 mm in diameter were aspirated using an 18-gauge needle and a 10 ml syringe. After being washed in Hepes buffered TALP medium, the cumulus-oocyte complexes were in vitro matured in 500µl of maturation medium at 38.5°C in 5% CO₂ in air at 100% humidity for 24-26 hours. After maturation, oocytes were inseminated with a final sperm concentration of 1 x10⁶ spermatozoa/ml in 500µl of fertilization medium.

**Sperm quality evaluation.**

The assessed sperm quality parameters were percentage of membrane intact spermatozoa, total and progressive motility, head to head agglutination (HHA) of the spermatozoa and acrosome reaction. The membrane integrity was determined by means of eosin-nigrosin staining and light microscopy (x 400). Two experienced people determined the total and progressive motility, and the HHA subjectively on a heated stage (37°C) using light microscopy (x 200). The motility was expressed in percentage, while the HHA was given a score from 0 (= no HHA) to 4 (all spermatozoa show HHA). The acrosome reaction was assessed before (t0) and after 5h of incubation (t2) by means of Pisum Sativum Agglutinin (PSA) staining with a Leica DMR fluorescence microscope (x 400) (Kawakami et al., 1993). Spermatozoa with a fluorescent green head indicated acrosome intact spermatozoa. Spermatozoa were considered as acrosome reacted when a fluorescent band was observed at the equatorial region.

**Evaluation of the fertilization / polyspermy rate**

After fertilization, oocytes were vortexed for 3 min in 2 ml of Hepes-TALP to remove excess sperm and/or cumulus cells, fixed overnight in 2% paraformaldehyde and 2% glutaraldehyde in phosphate buffered saline (PBS) solution without calcium and magnesium, and subsequently stained for 10 min with 1µg/ml of Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). The zygotes were mounted in 100% glycerol and evaluated by
means of a Leica DMR fluorescence microscope for fertilization and polyspermy. Successful fertilization was characterized by the presence of two pronuclei. The presence of more than two pronuclei indicated polyspermy.

**Experimental Design and Statistical Analysis**

*Experiment 1.*

Influence of whole blood and serum on sperm quality. After percoll separation, frozen-thawed semen was diluted in the media with 3 different concentrations of whole blood and serum to a concentration of $10 \times 10^6$/ml. The sperm quality was determined before (t0), after 30 min (t1) and after 5h (t2) incubation at room temperature. This experiment was repeated 4 times. Results of membrane integrity and acrosome reaction were analysed using a linear mixed effect model (S-Plus 2000, Cambridge, USA) including media with different blood and serum concentrations as fixed effect and sample as random effect. An autoregressive correlation structure of the first order (AR 1) was used to correct for dependency between repeated measurements on the same sample. The HHA was studied and descriptively recorded.

*Experiment 2.*

Effect of whole blood / serum on in vitro fertilizing capacity. After percoll separation, frozen-thawed semen was diluted in the media with 3 different concentrations of whole blood and serum to a concentration of $2 \times 10^6$/ml. Prior to insemination, spermatozoa were incubated for 30 min at 38.5°C in 5% CO$_2$ in air at maximum humidity. Twenty hours post insemination, the oocytes were fixed and stained with Hoechst 33342. In vitro fertilizing capacity and polyspermy were evaluated by means of fluorescence microscopy. This experiment was repeated 4 times. Results were analysed using logistic regression.

**RESULTS**

*Experiment 1.*

Effect of whole blood and serum on sperm quality: incubation of frozen-thawed spermatozoa for 30 min (data not shown) and 5h, respectively, in medium with blood and serum at room temperature, did not significantly affect membrane integrity ($P = 0.36$ and
Effect of whole blood and serum on bovine sperm quality and in vitro fertilizing capacity

0.89), total motility (P = 0.56 and 0.82), progressive motility (P = 0.25 and 0.07) or acrosome reaction (P = 0.57 and 0.24) (Table 1).

Table 1: Effect of 5h incubation (22°C) in media with whole blood and serum on the membrane integrity, total motility, progressive motility and acrosomal integrity of spermatozoa.

<table>
<thead>
<tr>
<th></th>
<th>Membrane intact (%)</th>
<th>Total motility (%)</th>
<th>Progressive motility (%)</th>
<th>Acrosomal integrity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>89.3 a</td>
<td>70.0 a</td>
<td>66.3 a</td>
<td>94.3 a</td>
</tr>
<tr>
<td>0.15%</td>
<td>90.7 a</td>
<td>63.8 a</td>
<td>48.8 a</td>
<td>96.0 a</td>
</tr>
<tr>
<td>1.5%</td>
<td>73.3 a</td>
<td>60.0 a</td>
<td>42.5 a</td>
<td>95.7 a</td>
</tr>
<tr>
<td>P = 0.89</td>
<td>P = 0.82</td>
<td>P = 0.07</td>
<td>P = 0.24</td>
<td></td>
</tr>
<tr>
<td>Blood serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>88.7 a</td>
<td>73.3 a</td>
<td>70.7 a</td>
<td>95.3 a</td>
</tr>
<tr>
<td>0.15%</td>
<td>87.3 a</td>
<td>76.7 a</td>
<td>60.0 a</td>
<td>96.3 a</td>
</tr>
<tr>
<td>1.5%</td>
<td>86.3 a</td>
<td>73.3 a</td>
<td>41.7 a</td>
<td>96.7 a</td>
</tr>
<tr>
<td>P = 0.36</td>
<td>P = 0.56</td>
<td>P = 0.25</td>
<td>P = 0.57</td>
<td></td>
</tr>
</tbody>
</table>

ab Values within the same column with different superscripts are significantly different (P < 0.05).

Increasing concentrations of blood did not affect the progressive motility (P = 0.25), while there was a trend that the progressive motility decreased with increasing concentrations of blood (P = 0.07) (Figure 1).

Also a non-significant decrease in total motility was found for the spermatozoa incubated in increasing concentrations of blood. The amount of head to head agglutination was the same for blood and serum. However, the amount of agglutination was clearly different between the 3 concentrations (0, 0.15 and 1.5%) (Figure 2). At t0, no agglutination was observed. At t1 and t2, agglutination was observed in medium containing 0.15% and 1.5% blood and serum (Figure 2).
Figure 2: Amount of agglutination (score 0-4) after 0 min, 30 min and 5h incubation in HEPES-Talp with 0%, 0.15% and 1.5% of whole blood or serum.

**Experiment 2.**

Effect of whole blood and serum on in vitro fertilizing capacity: increasing concentrations of blood and serum had a negative effect on the fertilizing capacity (P < 0.01). The fertilization rate decreased more with increasing serum concentrations than with increasing blood concentrations. Polyspermy was not significantly influenced by increased blood concentration (P = 0.30), but increasing serum concentrations significantly increased the polyspermy rates (P < 0.01) (Table 2).

Table 2: Effect of whole blood and serum on the percentage of normally fertilized oocytes (2 pronuclei) and percentage of polyspermy (> 2 pronuclei).

<table>
<thead>
<tr>
<th>Number of oocytes</th>
<th>Normally fertilization</th>
<th>Polyspermy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Whole blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>243</td>
<td>152</td>
</tr>
<tr>
<td>0.15%</td>
<td>272</td>
<td>138</td>
</tr>
<tr>
<td>1.5%</td>
<td>210</td>
<td>86</td>
</tr>
<tr>
<td>Blood serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>308</td>
<td>172</td>
</tr>
<tr>
<td>0.15%</td>
<td>328</td>
<td>140</td>
</tr>
<tr>
<td>1.5%</td>
<td>327</td>
<td>127</td>
</tr>
</tbody>
</table>

<sup>ab</sup> Values within the same column with different superscripts are significantly different (P < 0.05).

**DISCUSSION**

It is generally accepted that bovine semen quality can be affected by contaminations such as blood, urine, dirt or pus (Sigman and Lopez, 1993; Bar-Chama and Fisch, 1993; Chen et al., 1995; Griggers et al., 2001). However, in such cases the contamination originates from
disorders present in the bull’s genital tract. These disorders are mostly related to an infectious or inflammatory process. Under physiological conditions, low concentrations of blood may be present in the uterine fluid of the estrous cow at the moment of insemination (Andersen et al., 1985). However, little information is available on the effect of whole blood on sperm quality.

In our study we found that most parameters that are indicative of bovine sperm quality, were not affected by the presence of blood or serum, except for a significant effect on head to head agglutination (HHA). The HHA was more obvious in the presence of 1.5% blood or serum. In our study, total motility, progressive motility, membrane and acrosome integrity were not affected by the presence of blood or serum in the incubation medium. In human, 20-30% serum in an extender for fresh semen had a positive effect on at least one of the motility parameters (De Lamirande and Gagnon, 1991), which confirms our findings that serum admixture is not detrimental to sperm motility. However, in the presence of whole blood, which contains both plasma and cells such as leucocytes and erythrocytes, semen quality can be negatively affected. In the human, it has been demonstrated that the quality of fresh semen is negatively affected by the presence of white blood cells that produce reactive oxygen species, but not in the presence of RBCs (Wolff et al., 1990).

In pigs and dogs, admixture of whole blood (up to 5% and 10% respectively) in fresh diluted semen is not detrimental to semen quality (Le Roy, 1999; Rijsselaere et al., 2004). In these species blood may be present in the ejaculate due to trauma of the penis or the prepuce during semen collection, or to benign prostatic hypertrophy (Keenan, 1998; Johnston et al., 2001). In contrast, freezing of canine spermatozoa contaminated with blood has a detrimental effect on the post-thaw semen quality (Rijsselaere et al., 2004). In cattle, where frozen-thawed semen is used for insemination, contamination of blood during collection is not a problem because such samples are not used. Anderson et al. (1985) found $9.8 \pm 2.8 \times 10^6$ RBC/ml in raw uterine fluid at the day of estrus, which was comparable to the 0.15% concentration we used. Irrespective of service, many heifers and cows show a bright red sanguineous discharge at about 24-48 hours after heat. Blood in the uterine lumen originates mainly from the uterine caruncles, but it is not clearly defined at what moment of estrus the release of the blood into the uterine cavity occurs (Noakes et al., 2001b). The presence of blood in the uterine lumen is also more obvious when utero tubal junction (UTJ) insemination is performed, than when semen is deposited in the uterine body. After DIUI, traces of blood stick on the insemination pipette in 45% of the inseminations, while blood is seldom observed after classic insemination in the uterine body (Verberckmoes et al., 2002b).
Presence of blood in the uterine lumen in cattle at the time of insemination can be considered as a natural phenomenon as it is in dogs (Constantin et al., 1981). Therefore, as confirmed by our results, it should not be considered harmful to sperm quality. Despite the lack of effect upon several sperm quality characteristics, the presence of blood and serum did affect sperm fertilizing ability in a negative way, and serum did increase the number of polyspermic zygotes.

The effect of blood on in vivo fertility should not be overestimated, since it is known that fertilization occurs in the oviduct in the absence of blood. The HHA that occurred in the presence of blood and serum, is not necessarily detrimental for the fertilizing capacity of the spermatozoa. Agglutinated spermatozoa are characterized by a predominance of intact acrosomal membranes as judged by the presence of the apical ridge (Schroeder et al., 1990). This was confirmed in our study by means of PSA-staining. The possible negative effect of HHA on the in vivo fertility could be the result of temporarily mechanical inhibition of the spermatozoa (Schroeder et al., 1990) and their prolonged exposure to the environment of the uterus. In rabbits, spermatozoa engage immediately in HHA upon introduction in the uterine lumen (Senger and Saacke, 1976). In bovine, HHA in the reproductive tract has never been investigated. The physiological importance of this event, however, is not understood and the effect of HHA upon in vivo fertility is unknown.

In conclusion, no immediate effect of blood and serum was visible on several sperm quality parameters, except for an effect on HHA. However, blood and serum did have a negative effect on in vitro fertilizing capacity. When fresh or frozen semen is deposited into the uterine body, the presence of blood may interfere with in vivo fertility, if prolonged HHA prevents the spermatozoa to reach the sperm reservoir. This hypothesis needs further investigation. Application of a minimally harmful technique, by which spermatozoa are deposited close to the UTJ could help to reduce fertility problems by minimizing the contact between inseminated spermatozoa and blood.
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CHAPTER 10

GENERAL DISCUSSION
General discussion

Artificial insemination (AI) is the oldest and currently most common technique in assisted reproduction of animals. Initially the application of AI in cattle was forced by sanitary reasons and was performed with fresh semen. However, later economic reasons became the mainspring and since the optimization of cryopreservation of bovine semen around 1960, predominantly frozen-thawed semen has been used (Foote, 2002). The use of frozen semen facilitated distribution and enabled the creation of gene banks. However, despite the fact that semen of bovines is the least sensitive of all species to freezing damages, and despite the great efforts that have been made to optimize the freezing and thawing protocol, the optimal cell recovery is just over 50% (Vishwanath and Shannon, 2000). Currently, AI is performed in all farm animals: cattle, horses, sheep, goats, pigs, chickens, turkeys, rabbits, bees, …. However, the technical advancement in AI has been most successful in dairy cattle, which is especially due to the economic advantage gained when facing improved fertility rates and accelerated genetically progress (Vishwanath, 2003; Foote et al., 1956; Watson, 1990).

To optimize the use of top bulls, as many cows as possible have to be inseminated with a minimum number of spermatozoa and without a negative effect on the pregnancy rates. Therefore, insemination with low doses of semen is very interesting. Moreover, since the commercialisation of sex-sorted semen, which is damaged by the sexing procedure and merchandised in doses of 1.5 million frozen spermatozoa (Garner et al., 2001; Schenk et al., 1999), even more interest has arisen for low dose insemination.

Although the widely spread use of a limited number of highly valuable bulls can significantly increase the productivity of the herds, it also includes some important risks such as narrowing of the genetic diversity and rapid dispersal of masked genetic defects. To preserve the genetic diversity within the dairy cattle population it is important that decent breeding programs are set up and that the choice of sires is well considered. A regular alternation between different lines of bulls is therefore recommended. The progress which has been made in the last decade to trace genetic defects by means of genetic markers is promising. This method can offer a solution in the prevention of the dispersal of genetic defects (George et al., 1993; Takeda and Sugimoto, 2003). The combination of a well considered breeding program and further research to detect genetic defects will enable the use of a limited number of highly valuable bulls without major problems.
Two solutions which both have been investigated during our study have been stated to reduce the insemination dose without negative effects on fertility: 1) the use of fresh instead of frozen-thawed semen (Shannon, 1978; Foote and Parks, 1993), and 2) semen deposition close to the site of fertilization (Hunter, 2001).

One of the main restrictions of fresh semen is its limited shelf life. In New Zealand, where 95% of the semen is processed as a fresh product, semen can be stored for about 3 days in Caprogen® diluent (Thibier and Wagner, 2000). This limited storage period seriously hampers the ease of distribution. Many extenders have been developed for liquid storage of semen but, up to now, none of them has been capable of storing spermatozoa for more than 3 days without a drop in in vivo fertility (Foote, 1978). The development of a diluent which could store semen for 4 to 5 days would largely overcome the distribution problem (De Pauw et al., 2000). A new diluent (CEP-1 diluent), which was originally based on the composition of the bovine cauda epididymal plasma (CEP), has been developed in our laboratory for prolonged sperm preservation. Despite the fact that the CEP-1 diluent had the same ionic composition (Na, K, Cl, Ca, P, Mg), osmolarity and pH as the cauda epididymal plasma, no quiescent state or extended life span of the ejaculated spermatozoa was acquired (Chapter 3). The contact of the epididymal spermatozoa with the seminal plasma seems to induce an irreversible activation of the spermatozoa. Moreover, since sperm fertility declines much faster than sperm motility (Vishwanath and Shannon, 1997; 2000) and given the fact that after 2 days of incubation sperm motility was lower for spermatozoa stored in CEP-1 than in Tris diluent, it can be concluded that CEP-1 diluent was not appropriate for extended preservation of fresh bovine semen.

In order to improve the storage capacity of CEP-1 diluent, the ionic composition was modified and a new CEP-2 diluent was developed. In CEP-2 diluent the Ca, Mg, and inorganic P concentrations were increased, compared to CEP-1 diluent. The sperm storage capacity of CEP-2 diluent was compared with that of CEP-1 and Tris diluent (Chapter 4). The changes made to CEP-1 diluent had no effect on sperm membrane integrity and total motility, but the progressive sperm motility was clearly improved. In Chapter 4, also the effect of different polyols (sorbitol, glycerol, mannitol) and different concentrations of egg yolk were investigated. However, no difference in sperm quality was observed between spermatozoa stored in CEP-2 diluent in combination with different polyols and egg yolk concentrations.
Since the extended lifespan of spermatozoa in Caprogen® diluent can largely be attributed to the presence of catalase in the medium and storage under anaerobic conditions (Shannon, 1964; 1965; 1973), first the effect of catalase under aerobic and anaerobic conditions in CEP-2 diluent was evaluated. However, in contrast to Caprogen® addition of catalase to CEP-2 diluent had no beneficial effect on sperm quality, neither under aerobic nor under anaerobic conditions (Chapter 5). This can be due to the low storage temperature which not only may decrease enzymatic activity of catalase but also reduces the production of ROS to a minimal level. When only low ROS concentrations are present in the semen diluent, the protective effect of catalase may become unnoticed.

In Chapter 5 the in vitro storage capacity of CEP-2 diluent was compared with two other diluents which have been used for long term preservation of fresh bovine semen: Triladyl® (De Pauw et al., 2003) and Caprogen® (Shannon and Curson, 1984), and the best sperm motility characteristics were observed for spermatozoa stored in CEP-2 diluent. Spermatozoa stored in CEP-2 diluent moved significantly faster (VSL, BCF) and straighter (STR, LIN) than in Triladyl® and Caprogen® diluent. In the last two diluents increased percentages of hyperactivated spermatozoa were observed during subjective evaluation. This was confirmed by the increased amplitudes for lateral head displacement (ALH) obtained with Hamilton Thorne analysis. No significant differences in membrane and acrosome integrity or in mitochondrial membrane potential were observed between the three diluents during 6 days of storage. Although spermatozoa with intact membranes can be motile for an extended period of time, it does not mean that they are still capable of fertilization (Bartlett and Van Demark, 1962). Therefore in vitro fertilization experiments were performed (Chapter 5). Good fertilization rates (>50% or more) were obtained with frozen-thawed spermatozoa and with spermatozoa stored for 6 days at 5°C in CEP-2 and Caprogen® diluent. Significantly lower fertilization rates were obtained with spermatozoa stored in Triladyl® diluent. These results indicate that spermatozoa stored either in CEP-2 or in Caprogen® diluent have the same in vitro fertilizing capacity after 6 days of storage. The better sperm motility characteristics of spermatozoa stored for 6 days in CEP-2 diluent rather than in Triladyl® and Caprogen® diluent, and comparable IVF results of spermatozoa stored in CEP and Caprogen® diluent, indicate that the newly developed CEP-2 diluent can provide an alternative for the Caprogen® diluent which is at present assumed to be the best diluent for fresh bovine semen. To confirm the promising in vitro results in vivo trials are required. However, even if in vivo fertility is sufficiently preserved after 4 to 5 days of storage in CEP-2 diluent, the use of fresh bovine
semen will be of limited interest for most of the developed countries. Although the
distribution problem encountered with fresh semen can be partly solved by using the CEP-2
diluent, there still will remain a sanitary problem. To meet the sanitary requirements in
Europe and North America semen has to be free of a list of pathogenic agents. When fresh
semen is used, it is impossible to determine the sanitary status of the bull before distribution.
This is in contrast to frozen-thawed semen which is kept in quarantine until the sanitary status
of the bull at the moment of collection has been proven to be all right.

The main market for the CEP-2 diluent is expected to be the underdeveloped countries,
where the exact sanitary status of the bull is of low importance and also New Zealand. New
Zealand is an island where import of animals and related risk of import of diseases is very
limited. The sanitary status of all animals is similar within the whole country, which makes
determination of the sanitary status of indigenous bulls redundant. A subsidiary market for the
CEP-2 diluent can be established in the sperm sexing business. Currently only sex-sorted
semen originating from bulls owned by the semen sorting companies is available on the
market. However, when other companies which do not have a semen sorting laboratory have a
highly valuable bull of which the semen would be rewarding to be sexed it becomes very
difficult. Semen has to be collected at the AI centre, transported to the sorting laboratory and
finally distributed to different customers. Freezing semen both before and after sorting would
result in a tremendous decrease in sperm viability, and AI with such semen would be
unsuccessful. To minimize the decrease in sperm quality and loss before sorting, semen could
be collected and transported directly from the AI centre to the sorting laboratory in the CEP-2
diluent. This way of semen handling will result in similar pregnancy rates as currently
obtained with commercially available sex-sorted semen.

A further improvement which could be made to the CEP-2 diluent is the replacement of
egg yolk by synthetic recombinant proteins or phospholipids and to change it into a defined
medium. It is not unlikely that the use of substances of animal origin will be forbidden in the
near future due to risk of microbial or contagious material (e.g. prions) contamination.
Moreover, defined media are easier to standardize. In the Netherlands, field trials with
extenders in which egg yolk was replaced by soy bean extracts have been performed.
However, this resulted in a significant decrease of fertility rates (van Wagendonk-de Leeuw
et al., 2000).
Despite the fact that good in vitro results were obtained with the CEP-2 diluent, it is not certain that the in vivo results will also be satisfying. It is generally accepted that in vitro sperm quality assessment does not always give a good reflection of in vivo fertility. Several attempts have been made to predict in vivo bull fertility by means of a simple test which evaluates a single sperm characteristic such as sperm morphology (Barth, 1993), sperm motility (Stalhammar et al., 1994; Holt et al., 1997), or the presence of intact acrosomes (Cumming, 1995). Unfortunately none of these traits have been significantly correlated with in vivo sperm fertility. If a combination of different sperm traits is used, the reliability of the prediction of the fertilizing capacity can be increased (Amann and Hammerstedt, 1993; Farrell et al., 1998; Zhang et al., 1999; Rodriguez-Martinez, 2003). Recently a positive association has been shown between the number of bovine spermatozoa bound in cell cultures to 0.1 mm² oviductal epithelium and in vivo fertility (De Pauw et al., 2002). However, given the fact that this sperm-oviduct binding assay can only be performed in a well equipped laboratory, and that it is only reliable under certain conditions, it is not the Holy Grail for the prediction of in vivo fertility.

In our research we have investigated whether a correlation could be found between an in vitro sperm migration assay and in vivo sperm fertility (Chapter 6). In a sperm migration assay the sperm motility, morphology and capacitation are assessed simultaneously. In contrast to studies in humans (Alexander, 1981; Goldstein et al., 1982) we were not able to establish a positive correlation between the sperm migration distance in a migration medium with 1.35% methyl cellulose (MC) and the NRRs of 5 proven sires. The sperm migration distance after 15 and 30 min of incubation was even negatively related to the NRRs. In the present study we also showed that the progressive sperm motility was the best sperm quality parameter which was most correlated to the in vivo fertility of the bulls.

In the last decade, a great deal of research has been performed to evaluate the usefulness of flow-cytometry for a rapid and objective sperm assessment. The use of flow-cytometry for bovine sperm assessment and its value to predict in vivo fertility has been recently reviewed by Rodriguez-Martinez (2003). The main advantage of this technique is that several properties can be assessed simultaneously in a large number of spermatozoa. However, this highly sophisticated sperm analysis does not result in a better prediction of the in vivo fertility than subjective sperm motility assessment under a simple light microscope (Rodriguez-Martinez, 2003). Moreover, subjective sperm assessment can be rapidly performed under field conditions with minimal expenses. In conclusion, determination of the progressive sperm
motility remains the easiest and most important sperm characteristic which can be used to predict the in vivo fertility of bulls.

Besides using fresh instead of frozen-thawed semen, a second solution which has been stated to enable a reduction of the insemination dose without negative effects on fertility, is semen deposition closer to the oviduct (Hunter and Greve, 1998; Hunter, 2001). Semen deposition near the utero-tubal junction would result in a decreased loss of spermatozoa (Hawk, 1983; Larsson and Larsson, 1985; Mitchell et al., 1985; Nelson et al., 1987), and an increase in survival time of spermatozoa in the sperm-friendly environment of the isthmus (Suarez, 2001). In the past, the effect of deep insemination has been extensively investigated with varying success. However, semen deposition near the UTJ has never been performed in single ovulating cows with an insemination device with a flexible tip. This flexible tip is assumed to be the main characteristic which is required to enable proper UTJ insemination without damaging the endometrium. The increased contractility of the uterus and curling of the uterine horns at the moment of estrus hamper deep insemination with a rigid insemination device (Noakes et al., 2001). To enable UTJ insemination in a proper way, a new device with a flexible telescopic tip (Ghent device) has been developed and results obtained with this device are presented in Chapters 7 and 8. In Chapter 7 we have shown that the Ghent device, which is made of disposable materials, is both rigid enough to penetrate the cervix and flexible enough to follow the curves of the curled uterus. Moreover, the device can easily be handled by one person, is suitable under field conditions, and was proven to be not toxic for spermatozoa. Determination of the exact place of semen deposition can easily be performed by means of rectal palpation because the tip of the Ghent device can be easily located through the uterine wall. Since it has been shown that even conventional AI is not always that easy for inseminators (Peters et al., 1984), UTJ insemination which requires more skill of the inseminator may not be useful for do-it-yourself inseminators.

When pregnancy rates (PRs) obtained after AI with a full insemination dose (10-15 x 10^6) were compared, it was shown that with the Ghent device significantly higher pregnancy rates were obtained when semen was deposited near the UTJ than in the uterine body (P < 0.01). This can be due to the fact that the Ghent device is longer than the conventional insemination device, which complicates its fixation into the small uterine body (2 to 3 cm long) and increases the risk of endometrial damage. Especially the septum between both uterine horns is expected to be damaged. The contact with blood may induce head to head agglutination of the spermatozoa and hampering of the sperm motility (as discussed in Chapter 9). Moreover,
serious damage to the endometrium may alter the implantation of the embryo after descent into the uterus about 4 days after fertilization. The fact that no difference was found between UTJ insemination with the Ghent device and conventional insemination can be caused by the high insemination dose (10-15 x 10^6). It is assumed that semen deposition closer to the site of fertilization will predominantly have a beneficial effect on the pregnancy rates (PRs) when the number of spermatozoa or sperm viability is the limiting factor for fertilization. A question which can arise in cases of UTJ insemination with low quality semen is, whether the risk for congenital defects in the offspring is increased. However, in cases of UTJ insemination, there is still a natural selection of the most viable spermatozoa since they still have to pass the mucosal folds of the UTJ on their own forces. If DNA of the spermatozoa is seriously damaged, motility will be decreased and passage of the mucosal folds will become impossible. Moreover, even if such spermatozoa have been able to pass the mucosal folds they still need the capacity to fertilize before pregnancy is established. Finally, if low quality semen would induce more congenital defects in the offspring, this would already have been demonstrated in calves produced after in vitro fertilization, where spermatozoa and oocytes are brought together with a minimum of barriers.

Whether UTJ insemination could offer a solution for cows with fertility problems has not been investigated yet. When fertility problems are caused by an impaired uterine micro environment at the moment of insemination or by hampered sperm transport, e.g. by serosal adhesions, UTJ insemination could have a beneficial effect on the conception rates. However, in the majority of cases of subfertility it is more likely that not the number of spermatozoa or sperm quality are the limiting factors for fertilization, but especially the incorrect timing of the insemination because of estrus detection problems (Van Eerdenburg et al., 2002) or a low quality of the oocytes (Lonergan et al., 2001). In these cases, increasing the number of spermatozoa at the site of fertilization by semen deposition near the UTJ will not improve conception rates.

To investigate the hypothesis of Hunter (1998) which states that UTJ insemination would have a beneficial effect on fertility outcomes when the number of spermatozoa is the limiting factor for fertilization, field trials were performed in which the insemination doses were gradually decreased (Chapter 8). In these field trials the effect of UTJ insemination with the Ghent device was compared with uterine body insemination with the conventional device. However, decreasing the insemination dose from 12 to 2 x 10^6 frozen-thawed spermatozoa did not affect the PRs after AI with the Ghent device or with the conventional device. PRs
obtained after UTJ insemination did not differ significantly from PRs obtained after conventional AI, even not when the insemination dose was decreased to $2 \times 10^6$. These results indicate that with an insemination dose of $2 \times 10^6$ frozen-thawed spermatozoa the limiting sperm concentration is still not reached yet. This is in contrast to previous studies in which a clear decrease in fertility was observed at 15, 10 and $7.5 \times 10^6$ spermatozoa per insemination dose for low, moderately and highly fertile bulls (Uwland, 1984; Smidt and Zuidberg, 1988; Den Daas et al., 1998). The only difference between previous studies and the study that we performed was that in previous studies AIs were performed by technicians while in our study AIs were performed by veterinarians. This difference could be explained by the lack of accuracy by technicians to deposit semen into the uterine body so that often intracervical insemination is performed resulting in decreased fertility. In literature it has been reported that insufficient training of technicians can be an important reason for decreased fertility after conventional AI (Lopez-Gatius, 2000).

In order to show the possible beneficial effect of UTJ insemination compared to conventional AI a further decrease of the insemination dose is probably required.

In all field trials performed with the Ghent device, semen was deposited in the tip of both uterine horns. However, if the site of ovulation would be known, UTJ insemination could be performed ipsilaterally at the site of ovulation, and the insemination dose could be halved without a decrease in fertility. However, determination of the site of ovulation by rectal palpation can induce rupture of the preovulatory follicle and loss of the oocyte in the abdomen. Therefore, if ipsilateral insemination is considered, determination of the site of impending ovulation by ultrasound examination would be advisable. In cattle, Andersson et al. (2004) obtained similar pregnancy rates with $2 \times 10^6$ frozen-thawed spermatozoa after deposition in the middle of the horn ipsilateral to the site of ovulation with an embryo transfer pistolet (blue sheet, IMV) as with $15 \times 10^6$ frozen-thawed spermatozoa after deposition in the uterine body. Since semen can be deposited even closer to the UTJ with the Ghent device than with the embryo transfer pistolet, it is expected that similar pregnancy rates could be obtained with less than $2 \times 10^6$ frozen-thawed spermatozoa after ipsilateral insemination with the Ghent device. Moreover, in our last field trial we have already shown that deposition of $1 \times 10^6$ frozen-thawed spermatozoa in the tip of each uterine horn resulted in similar pregnancy rates as deposition of $12 \times 10^6$ frozen-thawed spermatozoa into the uterine body. In other single-ovulators such as horses and water buffaloes (Morris et al., 2000; Presicce et al., 2004) deposition of low doses of frozen-thawed spermatozoa ($1 - 3 \times 10^6$) near the UTJ ipsilateral to
the site of ovulation has been shown to be effective. In horses for example, similar pregnancy rates can be obtained with 0.5% of the conventional insemination dose when UTJ insemination is performed instead of conventional AI. When UTJ insemination ipsilateral to the site of ovulation would have the same effect in cattle as in horses no decrease in PR may be expected in cows after insemination with at 0.05 x 10⁶ frozen-thawed spermatozoa (= 0.5% of the conventional insemination dose). To confirm this hypothesis field trials with less than 2 x 10⁶ frozen-thawed spermatozoa are required. Also in pigs, a beneficial effect of UTJ has been demonstrated when low doses of poor fertile semen were used (Martinez et al., 2000, 2001; Grossfeld et al., 2003; Rath, 2003b). However, since pigs are multi-ovulators, half of the insemination dose had to be deposited at the left and half at the right UTJ.

To investigate whether more spermatozoa would be present in the oviduct after UTJ insemination than after conventional AI, an additional preliminary experiment was performed in which 7 cows were inseminated with the Ghent device ipsilateral to the site of impending ovulation and 6 cows with the conventional device in the uterine body. All cows were inseminated with 12 x 10⁶ frozen-thawed spermatozoa. However due to the low number of spermatozoa that could be recovered and the high variation between cows, no conclusions could be drawn from this experiment. Further research on a larger number of cows and with more accurate techniques to quantify the number of spermatozoa in the oviduct is required to evaluate this hypothesis. Moreover, the effects of timing of insemination, insemination technique and administration of different kind of drugs on the sperm population of the functional sperm reservoir in vivo are interesting topics to investigate more thoroughly. This research could elucidate the high variation in sperm numbers in the different segments of the female genital tract after insemination. More research is also required to determine the specific characteristics of the UTJ in order to clarify the high concentration of spermatozoa in this specific part of the bovine genital tract after insemination. Lines of thoughts are: specific structure of the narrow labyrinthine lumen (Hunter et al., 1991; Suarez, 2002), characteristics of the secretion (Hunter et al., 1991) and sperm receptors present at the UTJ (England and Pacey, 1998; Suarez, 2002).

In the field trial in which the feasibility of the Ghent device for UTJ insemination was assessed (Chapter 7), traces of blood were sometimes observed on the tip of the device. However, anecdotal records of the inseminators showed that the presence of blood on the tip of the insemination device did not always correspond with non-pregnancy. The presence of blood into the uterine lumen can be the result of damage to the endometrium, which is
hyperaemic at the moment of estrus, or can be the result of the late moment of insemination when blood is present in the uterine lumen, which is known as post-estrual bleeding (Noakes et al., 2001). In a preliminary trial timed AI was performed in 15 cows. Seven cows were inseminated with the Ghent device and 8 cows with the conventional device. Twelve hours after AI all cows were slaughtered and the endometrium was macroscopically and microscopically examined. However, in none of these cows clear endometrial damage could be observed macroscopically, and microscopically no increase of interstitial erythrocytes or neutrophils was detected in any of the cows. These results indicate that it is more likely that the blood sticking to the Ghent device was probably due to the rather late moment of insemination (post estrual bleeding) than to endometrial damage caused by the Ghent device.

In Chapter 9 the effect of blood and serum on sperm quality and in vitro fertilizing capacity is described. Sperm quality was not significantly affected by the presence of blood or serum. However, blood and serum did have a negative effect on in vitro fertilizing capacity. The effect of blood on in vivo fertility should not be overestimated, since it is known that fertilization occurs in the oviduct in the absence of blood. The induced head to head agglutination (HHA) in the presence of blood and serum, is not necessarily detrimental for the fertilizing capacity of the spermatozoa. However agglutination of spermatozoa hampers the progressive motility which is essential to pass through the UTJ, to move from the isthmus to the ampulla and to penetrate the oocyte (Hawk, 1987). Moreover, when spermatozoa are agglutinated and inhibited to pass the UTJ, their exposure to the uterine environment is prolonged. This may have a detrimental effect on spermatozoa since in pigs it has been shown that neutrophils enter the uterine lumen after insemination but not the lumen of the UTJ nor the oviductal isthmus (Hunter et al., 1987; Rodriguez-Martinez et al., 1990). On the other hand, it has been shown that head to head agglutination is only a temporarily mechanical inhibition of the spermatozoa (Schroeder et al., 1990).
CONCLUDING REMARKS

From the results described in this thesis, the following conclusions can be drawn:

1. CEP-1 diluent with the same ionic composition (Na⁺, K⁺, Cl⁻, Ca²⁺, P⁻, Mg²⁺) osmolarity and pH as the cauda epididymal plasma in the bull, has not the ability to bring ejaculated spermatozoa back into a quiescent state or to extend their life span.

2. CEP-2 diluent is a better diluent than Triladyl® and a good alternative to the Caprogen® diluent for long term storage of fresh bovine semen at 5°C.

3. The sperm migration test in 1.35% methyl cellulose cannot be used to predict in vivo bull fertility accurately.

4. No decrease in fertility is observed when the insemination dose is decreased from 12 to 2 million frozen-thawed spermatozoa, neither with the Ghent device nor with the conventional insemination device.

5. Further trials with even lower insemination doses are required to determine whether UTJ insemination with the Ghent device would be better than conventional insemination in cattle.

6. Blood and serum have no clear effect on several sperm quality parameters, except for head to head agglutination. However, blood and serum do have a negative effect on in vitro fertilizing capacity of spermatozoa.
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General discussion


CHAPTER 11

SUMMARY – SAMENVATTING
Summary

The goal of the AI-industry is to maximize the number of offspring produced by selected genetically superior bulls. This can be accomplished by decreasing the number of spermatozoa per insemination dose without a reduction in fertility. Recently it has become possible to sort bovine semen in X- and Y-bearing spermatozoa by means of flowcytometry. However, due to the high costs of the sexing procedure, a dose of sexed semen contains 1 to 2 million frozen-thawed spermatozoa, which is only 10% of a conventional insemination dose. Moreover, the sexing procedure has a detrimental effect on sperm quality. To improve fertility rates with low dose insemination and less fertile semen (i.e. sex-sorted semen), two solutions are possible; 1) insemination with fresh instead of frozen-thawed semen, and 2) semen deposition close to the site of fertilization.

To reach these goals, the two most important aims of this thesis were: 1) to develop a diluent in which fresh bovine semen could be stored for several days without a reduction in fertilizing capacity (Chapters 3-5) (Aim 1), and to investigate whether semen deposition near the utero-tubal junction by means of a new insemination device could improve fertility rates with low insemination doses (Chapters 7 and 8) (Aim 2). A minor aim of this study was to determine whether the in vitro results, as obtained for CEP-2 diluent, could be related with in vivo fertility (Aim 3). Therefore a sperm migration assay was evaluated (Chapter 6). Finally, the effect of blood and serum on bovine sperm quality was investigated, since in some cases during the field trial blood was sticking on the insemination device (Chapter 9) (Aim 4).

In Chapter 2, the differences in indications and applications of intra-uterine insemination in farm animals and humans are described. Moreover, in this chapter it is illustrated that due to the introduction of flowcytometrical semen sorting in cattle a similar situation of infertility has arisen as in humans.

In Chapter 3 the methods for in vitro and in vivo collection of cauda epididymal plasma (CEP) in the ram and bull are discussed, and the biochemical composition of the collected CEP is given. A semen diluent (CEP-1) with the same ionic composition, pH and osmolarity as the bovine CEP was developed and compared with the standard Tris diluent for extended preservation of fresh ejaculated semen. However, spermatozoa stored in the CEP-1 diluent did not acquire a quiescent state nor had an extended shelf life.
Due to the poor storage results obtained with the CEP-1 diluent, its ionic composition was modified (CEP-2) and in Chapter 4 the storage capacity of the CEP-2 diluent was compared with that of CEP-1 and Tris diluent. Subsequently, the effect of addition of different polyols (sorbitol, glycerol, mannitol) and egg yolk concentrations (5, 10 and 20%) to the CEP-2 diluent was assessed. Finally, CEP-2 diluent with 10% egg yolk and 1g/L sorbitol turned out to be an appropriate diluent for extended preservation of fresh bovine semen at 5°C.

In New Zealand, where 95% of the semen used for artificial insemination in cattle is processed as fresh semen, Caprogen® is used as diluent. This Caprogen® diluent is considered as being the most appropriate diluent for the storage of bovine liquid semen. The main characteristics of the Caprogen® diluent are: 1) the presence of catalase, and 2) the storage under N₂ gas. Spermatozoa can be stored for 3 days in Caprogen® diluent and used for AI without a significant decrease in fertility. In order to investigate whether catalase and storage under N₂ gas also have a beneficial effect on the quality and in vitro fertilizing capacity of spermatozoa stored in CEP-2 diluent, further experiments on fresh semen preservation were performed (Chapter 5). However, addition of 4.5 mg/ml catalase to CEP-2 diluent under aerobic and anaerobic conditions had no effect on sperm quality. Spermatozoa stored in CEP-2 diluent moved faster and straighter than spermatozoa stored in Triladyl® or Caprogen® diluent. The in vitro fertilizing capacity of spermatozoa stored for 6 days at 5°C in CEP-2 and Caprogen® diluent was good, while it was significantly worse for spermatozoa stored in Triladyl® diluent. Based on the in vitro results, the CEP-2 diluent is a better diluent than Triladyl® and could serve as a good alternative to the Caprogen® diluent for long term storage of fresh bovine semen at 5°C.

Notwithstanding in vitro sperm assays can give an indication of in vivo fertility, up to now, no assay has been proven to be successful in predicting in vivo fertility accurately. In human infertility studies sperm migration assays have been extensively refined and validated, while the application of sperm migration assays to predict bovine fertility has been very limited. Moreover, a clear relation between sperm migration distance and in vivo bull fertility has never been demonstrated. In Chapter 6, a synthetic medium based upon methyl cellulose was tested for its suitability to serve as a migration medium for frozen-thawed bovine spermatozoa and the relation between sperm migration capacity and NRR was assessed. Despite the relatively high repeatability of the sperm migration assay after 30 min incubation in medium with 1.35% methyl cellulose, no relation was found between the sperm migration
distance and the 56d NRR. Therefore, the evaluated sperm migration assay cannot be used to predict in vivo bull fertility accurately.

Apart from the use of fresh bovine semen instead of frozen-thawed semen, semen deposition near the UTJ has been suggested as an alternative technique to increase the number of descendents of genetically highly valuable bulls. Hence a new artificial insemination device for semen deposition near the UTJ in cattle (Ghent device) has been developed at our department. In Chapter 7 this Ghent device and the effect of the insemination catheter on sperm quality is described. Moreover, in a field trial the efficacy of the Ghent device was assessed. No toxic effect of the insemination catheter on sperm quality or fertilizing capacity was found. In the field trial it was shown that the Ghent device was suitable for UTJ insemination in dairy cattle under field conditions. Moreover, when the Ghent device was used for AI, better pregnancy rates were obtained after deposition near the UTJ than into the uterine body.

In Chapter 8, more field trials are described in which the Ghent device is compared to the conventional Cassou insemination device using decreasing insemination doses (8, 4, and 2 million frozen-thawed spermatozoa). However, no decrease in fertility was observed when the insemination dose was decreased from 12 to 2 million frozen-thawed spermatozoa, neither with the Ghent device nor with the conventional insemination device. Field trials with even lower insemination doses are required to determine whether UTJ insemination with the Ghent device is effectively better than the conventional insemination method in cattle.

In the field trial in which the practical use of the Ghent device for UTJ insemination was assessed (Chapters 7 and 8), it was found that in some cases blood was sticking on the tip of the device. In Chapter 9, the effect of blood and serum on sperm quality and in vitro fertilizing capacity is described. Sperm quality as assessed by total motility, progressive motility, membrane integrity and acrosomal status was not affected by the presence of blood or serum. However, the prevalence of head-to-head agglutination was increased in the presence of blood and serum. Moreover, the in vitro fertilizing capacity of spermatozoa stored in medium containing blood or serum was decreased.
The general discussion and conclusions are presented in Chapter 10.

From the results described in this thesis, the following conclusions can be drawn:

1. CEP-1 diluent with the same ionic composition (Na, K, Cl, Ca, P, Mg) osmolarity and pH as the cauda epididymal plasma in the bull, has not the ability to bring ejaculated spermatozoa back into a quiescent state or to extend their life span.

2. In vitro results indicate that CEP-2 diluent is a better semen diluent than Triladyl® and a good alternative to the Caprogen® diluent for long term storage of fresh bovine semen at 5°C.

3. The sperm migration test in 1.35% methyl cellulose can not be used to predict in vivo bull fertility accurately.

4. No decrease in fertility was observed in cows when the insemination dose was decreased from 12 to 2 million frozen-thawed spermatozoa, neither with the Ghent device nor with the conventional insemination device.

5. Further trials with even lower insemination doses are required to determine whether UTJ insemination with the Ghent device would give better results than conventional insemination.

6. Blood and serum have no clear effect on several sperm quality parameters, except for head to head agglutination. However, blood and serum do have a negative effect on in vitro fertilizing capacity of spermatozoa.
Samenvatting

Het doel van KI-verenigingen en fokkerij organisaties is het aantal nakomelingen van genetisch hoogwaardige stieren te maximaliseren. Dit doel zou bereikt kunnen worden door het aantal spermatozoa per inseminatiedosis te verminderen maar dan mag dit niet ten koste gaan van de fertiliteit. Onlangs is men erin geslaagd om stierensperma te scheiden in X- en Y-dragende spermacellen met behulp van flowcytometrie. Toch zijn er enkele nadelen aan het scheiden van sperma. Wegens de hoge kostprijs van de scheidingsprocedure bevat een inseminatiedosis slechts 1 à 2 miljoen spermatozoa, wat slechts 10% van de standaard inseminatiedosis is. Bovendien heeft het scheidingsproces een negatief effect op de spermakwaliteit. Om het probleem van verminderde fertiliteit na inseminatie met lage inseminatiedoses of met minder goed vruchtbaar sperma (bv gesekst sperma) op te lossen, zijn er twee mogelijkheden: 1) inseminatie met vers sperma in plaats van diepvries sperma, en 2) sperma deponeren dicht bij de plaats van bevruchting (isthmus van de eileider).

De twee belangrijkste doelstellingen van het in dit proefschrift beschreven onderzoek waren: 1) de ontwikkeling van een verdunner voor het bewaren van vers stierensperma gedurende meerdere dagen zonder dat er een vermindering van de bevruuchtingscapaciteit optreedt (Hoofdstuk 3-5), en 2) het onderzoeken of spermadepositie nabij de utero-tubale junctie met behulp van een nieuwe inseminatiepipet de mogelijkheid biedt om de resultaten na KI met lage inseminatiedoses te verbeteren (Hoofdstuk 7 en 8). Een bijkomstig doel van deze studie was na te gaan of de in vitro resultaten die verkregen werden met de CEP-2 verdunner op één of andere manier met in vivo resultaten konden gerelateerd worden. Hiervoor werd gebruik gemaakt van een spermamigratietest (Hoofdstuk 6). Aangezien er tijdens de veldproeven af en toe een spoortje bloed aan de pipet kleefde, werd het effect van bloed en serum op de kwaliteit van het sperma onderzocht (Hoofdstuk 9) (doelstelling 4).

In Hoofdstuk 2 worden de verschillende indicaties en toepassingen voor het uitvoeren van intra-uteriene inseminatie bij huisdieren en bij de mens beschreven. Bovendien wordt in dit hoofdstuk geschetst hoe de introductie van het flowcytometrisch scheiden van stierensperma geleid heeft tot een vergelijkbaar probleem als ook bestaat bij de mens, namelijk sperma dat van onvoldoende kwaliteit is om de eicel te bevruchten.

In Hoofdstuk 3 worden de verschillende methoden beschreven die kunnen gebruikt worden om cauda epididymaal plasma (CEP) te verzamelen onder in vitro en in vivo condities.
bij de ram en de stier. Eveneens wordt in dit hoofdstuk de biochemische samenstelling van het onderzochte CEP beschreven. Op basis van de ionaire samenstelling, de pH en osmolariteit van het CEP werd een spermaverdunner (CEP-1) ontwikkeld en vergeleken met de standaard Tris-verdunner voor de langdurige bewaring van vers geëjaculeerd stierensperma. Toch kon het sperma dat in de CEP-1 verdunner bewaard werd niet opnieuw in een “rust” stadium gebracht worden. Eveneens kon de levensduur van het sperma dat in de CEP-1 verdunner bewaard werd niet verlengd worden.

Wegens de tegenvallende resultaten die verkregen werden met de CEP-1 verdunner, werd de ionaire samenstelling aangepast (CEP-2) en werd in Hoofdstuk 4 de bewaarcapaciteit van de CEP-2 verdunner vergeleken met die van de CEP-1 en Tris verdunner. Vervolgens werd het effect van toevoegen van verschillende polyolen (sorbitol, glycerol, mannitol) en van verschillende eigeelconcentraties (5, 10 en 20%) aan de CEP-2 verdunner uitgetest. Finaal bleek de CEP-2 verdunner met 1g/l sorbitol en 10% eigeel een geschikte verdunner te zijn voor de langdurige bewaring van vers stierensperma bij 5°C.

In Nieuw Zeeland wordt 95% van de koeien geïnsemineerd met vers bewaard sperma. De bewaring van dit sperma gebeurt in de Caprogen® verdunner. Deze Caprogen® verdunner wordt beschouwd als de meest complete verdunner voor de bewaring van vers stierensperma. De belangrijkste kenmerken van de Caprogen® verdunner zijn de aanwezigheid van catalase, en de bewaring onder N₂ gas. In de Caprogen® verdunner kan het sperma gedurende 3 dagen bewaard worden en vervolgens gebruikt worden voor KI zonder dat er een significante daling optreedt in de drachtigheidsresultaten. Om na te gaan of catalase en bewaring onder N₂ gas ook een positief effect heeft op de spermakwaliteit en op het in vitro bevruchtend vermogen van de spermatozoa die in de CEP-2 verdunner bewaard worden, werden enkele experimenten uitgevoerd (Hooftstuk 5). Uit deze experimenten bleek dat toevoeging van 4,5 mg/ml catalase aan de CEP-2 verdunner noch de bewaring onder aërobe of anaërobe omstandigheden geen effect hadden op de spermakwaliteit. De spermacellen bewaard in de CEP-2 verdunner bewogen echter wel sneller en rechtlijniger dan deze bewaard in Triladyl® of Caprogen® verdunner. Het in vitro bevruchttend vermogen van spermacellen die gedurende 6 dagen bewaard werden bij 5°C in CEP-2 en Caprogen® verdunner was goed, terwijl dit significant slechter was voor spermacellen die bewaard werden in Triladyl verdunner. Op basis van de in vitro resultaten kunnen we besluiten dat de CEP-2 verdunner beter is dan de Triladyl verdunner en dat hij kan dienen als een goed alternatief voor de Caprogen® verdunner in het kader van de langdurige bewaring van vers stierensperma bij 5°C.
Niettegenstaande het feit dat verschillende in vitro spermatesten een goede indicator zouden kunnen zijn voor het in vivo bevruchtend vermogen van sperma, is er tot op heden nog geen enkele die succesvol blijkt te zijn om de in vivo fertiliteit accuraat te voorspellen. Bij de mens is het gebruik van spermamigratietesten uitgebreid bestudeerd. Het gebruik van dergelijke spermamigratietesten is bij rundvee daarentegen nooit goed onderzocht. Bovendien is er bij rundvee nooit een duidelijke correlatie aangetoond tussen de spermamigratieafstand en het in vivo bevruchtend vermogen van de stier. In Hoofdstuk 6 werd een synthetisch medium op basis van methylcellulose (MC) getest om na te gaan of dit kon dienen als migratiemedium voor diepvries-stiersperma. Eveneens werd de relatie tussen de spermamigratiecapaciteit en de non-return rate (NRR) beoordeeld. Ondanks de relatief hoge herhaalbaarheid van de spermamigratietest na 30 min incubatie in medium met 1.35% MC, werd er geen verband gevonden tussen de spermamigratieafstand en de 56-dagen NRR. Bijgevolg kan hieruit besloten worden dat de spermamigratietest niet kan gebruikt worden voor het accuraat voorspellen van de in vivo fertiliteit van stiersperma.

Naast het gebruik van vers in plaats van diepvries-stiersperma, wordt verondersteld dat spermadepositie nabij de UTJ een alternatief zou kunnen zijn om het aantal nakomelingen van genetisch hoogwaardige stieren te verhogen. Om het effect van UTJ inseminatie uit te testen werd aan onze vakgroep een nieuwe inseminatiepipet (Ghent device) ontwikkeld. In Hoofdstuk 7 werden het “Ghent device” en het effect van deze pipet op de spermakwaliteit beschreven. Bovendien werd de efficaciteit van het “Ghent device” onderzocht in een veldstudie. Uit het onderzoek bleek dat de inseminatiecatheter niet schadelijk was voor de spermakwaliteit of voor het in vitro bevruchtend vermogen. De veldstudie toonde aan dat het Ghent device gebruikt kon worden voor het uitvoeren van utero-tubale junctie (UTJ) inseminaties onder veldomstandigheden. Bovendien werd vastgesteld dat betere drachtigheidsresultaten werden bekomen na deponeren van sperma nabij de UTJ dan in het corpus uteri wanneer de inseminaties werden uitgevoerd met het “Ghent device”.

In Hoofdstuk 8 werden meerdere veldproeven met verlaagde inseminatiedoses (8, 4 en 2 miljoen ontdooide diepgeworven spermacellen) beschreven waarin resultaten verkregen met het “Ghent device” vergeleken werden met die van de conventionele inseminatiepipet. Er werd geen daling van de fertiliteit waargenomen wanneer de inseminatiedoses verlaagd werden, noch met het “Ghent device” noch met de conventionele inseminatiepipet. Om na te gaan of UTJ inseminatie met het “Ghent device” effectief beter is dan inseminatie met de conventionele inseminatietechniek zijn veldproeven met nog lagere inseminatiedoses nodig.
In de veldproeven waarin de bruikbaarheid van het “Ghent device” voor UTJ inseminatie onderzocht werd (Hoofdstuk 7 en 8) werd er af en toe een spoortje bloed waargenomen aan de punt van de inseminatiepipet. Daarom werd het effect van bloed en serum op de spermakwaliteit en op het in vitro bevruchtend vermogen onderzocht in Hoofdstuk 9. De spermakwaliteit die beoordeeld werd op basis van totale motiliteit, progressieve motiliteit, membraan intactheid en acrosoomstatus werd niet aangetast door de aanwezigheid van bloed of serum. Toch steeg de prevalentie van kop-aan-kop agglutinatie wanneer sperma in contact kwam met bloed of serum. Bovendien was het in vitro bevruchtend vermogen van spermacellen die bewaard werden in medium dat bloed of serum bevatte verminderd.

In Hoofdstuk 10 bevat de algemene discussie en de conclusies.

Op basis van de resultaten die in dit onderzoek bekomen werden, kunnen de volgende conclusies getrokken worden:

1. Met de CEP-1 verdunner die dezelfde ionaire samenstelling (Na⁺, K⁺, Cl⁻, Ca⁺, P⁻, Mg²⁺), osmolariteit en pH heeft als het cauda epididymaal plasma van de stier kunnen geëjaculeerde spermacellen niet opnieuw in een ruststadium worden gebracht. Ook kan de overlevensduur van het sperma daarmee niet verlengd worden.

2. Uit in vitro resultaten blijkt dat de CEP-2 verdunner een betere spermaverdunner is dan Trilayl® en een good alternatief kan zijn voor de Caprogen® verdunner om vers stierensperma langdurig te bewaren bij 5°C.

3. De spermamigratietest in 1,35% methylcellulose kan niet gebruikt worden om de in vivo fertiliteit van een stierensperma accuraat te voorspellen.

4. Zelfs met de sterk verlaagde inseminatiedoses werd geen vermindering van het drachtigheidspercentage vastgesteld, noch met het “Ghent device” noch met de conventionele inseminatiepipet.

5. Proeven met nog lagere inseminatiedoses zijn nodig om na te gaan of UTJ inseminatie met het “Ghent device” betere resultaten geeft dan de conventionele inseminatietechniek.

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DP.
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