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Improved Fertility of Flowcytometrically Sex Selected Bull Spermatozoa

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To my Family

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List of abbreviations

AI	Artificial Insemination
AO	Antioxidants
ATP	Adenosin Triphosphat
BSA	Bovine Serum Albumin
CFDA	Carboxyfluorescein Diacetate
cm	centimetre
DIU-AI	Deep Intra-Uterine Insemination
DNA	Deoxyribonucleic Acid
ET	Embryo Transfer
F/T	Frozen/Thawed
FISH	Fluorescence in Situ Hybridization
FITC-PNA	fluorescein isothiocyanate labeled peanut from Arachis hypogaea
GnRH	Gonadotropin-Releasing Hormone
h	hours
ICSI	Intra-Cytoplasmatic Sperm Injection
IVF	In Vitro Fertilization
kg	kilogram
L	Litre
LN_2	Liquid Nitrogen
LPC	L-α-Lysophosphatidylcholine
MAS	Morphological Abnormal Spermatozoa
mg	milligram
min.	minute
mL	millilitre
mM	milimol
n	number
PBS	Phosphate Buffered Salts
PCR	Polymerase Chain Reaction
$PGF_{2\alpha}$	Prostaglandin 2α
PI	Propidium Iodide
PVP	Polyvinil Pyrrolidone
ROS	Reactive Oxygen Species
SD	Standard Deviation
sec.	second
TDF	Testis-Determining Factor
TRIS	Tris-hydroxymethyl-aminomethane
UV	Ultraviolet
W	Watt
ZFY	Zinc-finger gene on the Y-chromosome
µL	micro litre

1 Introduction

Flowcytometrical sorting of mammalian spermatozoa has been proven as a powerful tool for increasing the benefits in animal production, for the genetic improvement of farm animals, for the control of sex-linked disease in humans and in wildlife for the re-population of endangered species. Although different techniques for sex separation of spermatozoa were developed in the past, the Beltsville sperm sexing technology is the only technique that enables the birth of offspring with desired sex in more than 90 %.

Several major improvements, like the invention of high speed cell sorting and improved orientation of cells in front of the laser, have been made in the past years. Nevertheless, the limitations of the technology due to the principle of single sperm cell analysis have to be recognized and even with further technical improvements it is unlikely that the output will reach the amount of a normal insemination dosage. Bovine sexed sorted spermatozoa are in combination with other biotechnologies already commercially available, but intensive research is required to provide a higher quality of sorted spermatozoa for artificial insemination. The High speed flowcytometer enables the production of 15 millions sex sorted spermatozoa per hour. With selected bulls, acceptable pregnancy rates in cattle are achievable after artificial insemination with only 2 millions of unsorted spermatozoa. This amount would be small enough for commercial application of sperm sorting technology in the bovine. However, also in this species the main limitation for commercial application remains the quality, short lifespan and consequently reduced fertility of sex sorted spermatozoa compared to unsorted semen.

Therefore the objective of the present thesis was to improve the pre- and post sort processing of the semen in order to maintain sperm integrity and viability in fresh and frozen/thawed semen samples.

2 Review of literature

2.1 Sex predetermination - historical development

Since ancient times, sex determination has been of interest for man. First attempts to control sex were described by philosophers in pre-ancient Greece. DEMOCRITUS OF ABADERA (460-370 BC) believed that males originate from the right testicle and females from the left. Further, it was said that males developed more often in the right and females in the left uterine horn. According to this presumption, recommendations for human coitus and in some instances even castration of "unwanted" testis were performed in order to obtain a child of the desired sex. A first description of "Sperma" came from HIPPOCRATES (460-377 BC). He believed that "Sperma" plays a key role in the development of a child. If "Sperma", interestingly produced from both sexes, was strong from both parents, then a male would be born. In contrast to that, "weak Sperma" was correlated with the development of a girl. Different selective strategies for gender pre-selection were developed on these assumptions and claims of other philosophers. Although ANTON VAN LEEUWEHOEK (1677) described spermatozoa for the first time using an improved microscope, real scientific approaches of gender pre-selection were first undertaken in the 20th century with the development of more sophisticated instruments (HUNTER 1995).

2.2 Natural mechanism of sex determination

Nature has developed several systems to maintain a balanced distribution of genetic information including sex related mechanisms within the population. Major impacts on the development of such systems are related to environmental dependence of a species. For example, in many reptiles, temperature depending enzymes regulate the sex of offspring (DORIZZI et al. 1996; GABRIEL et al. 2001; PIEAU et al. 2001). However, in some reptile species, sex is determined chromosomally depending on the combination of sex chromosomes (ZW vs. ZZ) as found in lizards and turtles (CORIAT et al. 1994).

In mammals and avian species, the primary regulation of sex determination depends on chromosomal information only. Whereas in the latter, female gametes are heterogametic, mammalian spermatozoa carry either a Y- or X-chromosome

(JACOBS and STRONG 1959; MCLAREN and MONK 1981). The Y-chromosome carries the genetic information for a testis-determining factor (TDF) that initiates the formation of testicular material in the primitive genital ridge. After that, the morphological and functional development of the male genital apparatus is mainly hormonal dependent and suppresses in parallel development of the female genital tract. A precise localization of the TDF region and the genes that are included in sex differentiation were investigated comparatively between XY and XX males. DNA of these individuals include different amounts of the Y-chromosome and their analysis allowed to map the short arm of the Y-chromosome (MULLER et al. 1986). Further analysis of this region revealed that more genes are involved in sex differentiation. Cloning and screening of this region first discovered the zinc-finger gene on the Y-chromosome (ZFY) believed to be the testis determination factor (TDF) (PAGE et al. 1987). However, evidence was provided that ZFY cannot be the TDF, because some of XX males were ZFY negative (PALMER et al. 1989). Another candidate for TDF is the "sex region on the Y-chromosome" (SRY), which was discovered shortly after ZFY. Expression studies, mutational analysis of SRY in XY females, production of transgenic mice, and biochemical analysis provided further evidence that SRY is TDF (HARLEY et al. 1992; JAGER et al. 1990; KOOPMAN et al. 1990; KOOPMAN et al. 1991).

2.3 Techniques to identify sex-related characteristics of spermatozoa

The most effective way to influence sex ratios in offspring is to determine the sex before fertilization and therefore separate populations of X- and Y-chromosome bearing spermatozoa. Several techniques based on principals such as velocity (BEAL et al. 1984; BEERNINK and ERICSSON 1982; DMOWSKI et al. 1979; ERICSSON et al. 1973; ZAVOS 1985), density (BHATTACHARYA et al. 1966; BHATTACHARYA 1962; FLAHERTY et al. 1997; KANEKO et al. 1983; KOBAYASHI et al. 2004; LOPEZ et al. 1993; PYRZAK 1994; QUINLIVAN et al. 1982; ROHDE et al. 1975; ROSS et al. 1975; SCHILLING and THORMAEHLEN 1977; SHASTRY et al. 1977; VIDAL et al. 1993; WANG et al. 1994b; WANG et al. 1994a), electric surface charge (BLOTTNER et al. 1994; ENGELMANN et al. 1988; MANGER et al. 1997; SEVINC 1968; SHIRAI et al. 1974; SHISHITO et al. 1974; UWLAND and WILLEMS 1975), and immunologically relevant structures (ALI et al. 1990;

ERICKSON et al. 1981; HANCOCK 1978; HENDRIKSEN et al. 1993; SILLS et al. 1998), have been developed and tested. Neither of these methods was able to produce significant separation of fertile sperm populations, or was not repeatable.

The only method known so far uses the relative difference in DNA content of X- and Y- chromosome bearing spermatozoa. By mid of the last century research on DNA indicated that its amount differs among sex chromosomes. It led to the idea to identify spermatozoa on this basis. MORUZZI (1979) showed that the X-chromosome carries more DNA than the Y-chromosome and autosomal cells have identical DNA content.

2.4 Development of flowcytometrical sperm sorting

At the same time, flowcytometry was developed (SPRENGER et al. 1971) and GLEDHILL et al. (1976) reported about first experiments of flowcytometrical sperm analysis. Unfortunately, these experiments failed, until the problem of flat cell orientation was first solved after employing two sheath-liquid streams in the analysis of chicken erythrocytes (FULWYLER 1977). The adaptation of the injection tube to wedge shape and inclusion of a second light detector were necessary to gain higher resolution for analysis of flat cells (DEAN et al. 1978; STOVEL et al. 1978). Accordingly, PINKEL et al. (1982) modified their system to oriented spermatozoa in front of the laser beam. These improvements of the flowcytometer were the prerequisites to detect differences in DNA content of X- and Y- chromosome bearing spermatozoa, first reported by GARNER et al. (1983). In their initial projects, spermatozoa had to be fixed in ethanol for flowcytometrical analysis after being labelled with the fluorochrome 4'-6-diamidino-2-phenylindole (DAPI) in order to achieve two different peaks representing X- and Y- sperm populations and to quantify the difference in DNA content between spermatozoa for bull (3.8 %), boar (3.7 %), ram (4.1 %) and rabbit (3.9 %). Due to the labelling process, spermatozoa had no fertilizing abilities after sorting, but the experiment approved the differentiation of X- and Y-chromosome bearing spermatozoa, opening a new field in biotechnology.

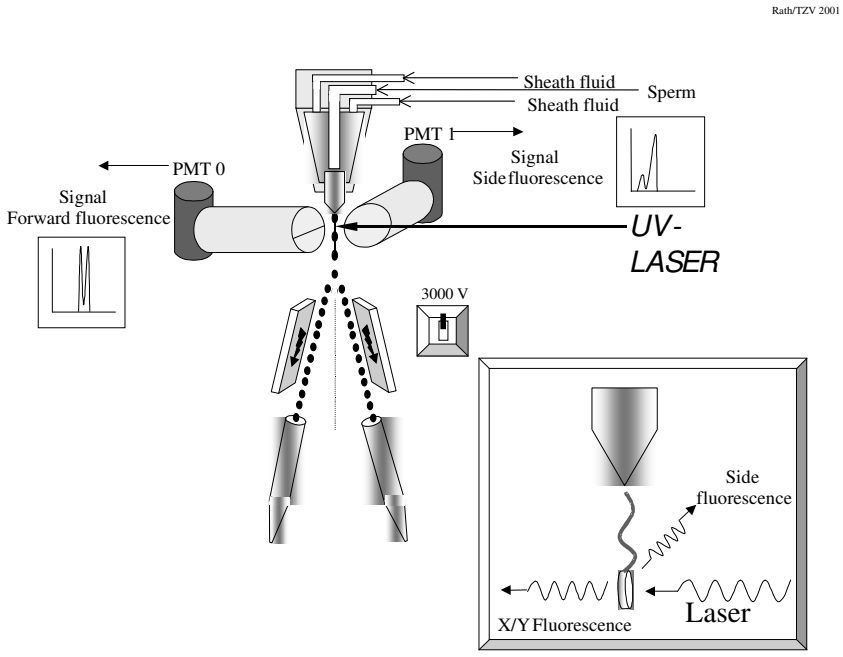
Several further modifications on the flowcytometer and in sample preparation were necessary to obtain a higher resolution. JOHNSON and PINKEL (1986) modified a Coulter EPICS V flowcytometer, adding a second fluorescence detector at 0° and

developed a bevelled tip for the sample injection tube. Ethanol was still used for the fixation of the spermatozoa, but labelling of spermatozoa was performed now with Hoechst 33342, allowing specific and relatively uniform staining of the DNA (JOHNSON et al. 1987a; JOHNSON et al. 1987b). Despite evidence of its detrimental effect on cell growth (ERBA et al. 1988), Hoechst 33342 was chosen as the least toxic from all DNA fluorescent stains that have potentials to penetrate the plasma membrane of living cells (JOHNSON and CLARKE 1988). The stain selectively binds to A-T rich regions of DNA and enables the detection of small differences in DNA content.

The sorting process with a modified standard flowcytometer was relatively slow and allowed separation of about 55 sperm heads/second. In 1988 first evidence was seen that flowcytometrically sorted spermatozoa from domestic animals were able to decondense and form a pronucleus after sperm injection into a hamster oocyte (JOHNSON and CLARKE 1988). Births of the first animals inseminated with viable sorted spermatozoa were reported in the same year (MORRELL et al. 1988). A year later surgical inseminations with sorted spermatozoa into the uterus of does resulted in the birth of offspring with a significant shift in sex ratio. Among 37 offspring the proper sex was observed in 94 % and 81 % of animals after insemination with X- and Y-chromosome bearing spermatozoa, respectively (JOHNSON et al. 1989).

Sperm sorting with a flowcytometer requires several technical modifications of a standard apparatus to measure DNA differences in X- and Y-bearing spermatozoa. The flowcytometer has to be equipped with a 5 W UV-light, water-cooled argon laser. This may change now, as the first solid UV-lasers appeared on the market. Besides longer lifetime their signal can be pulsed in order to minimize negative effects on sperm integrity. Until 1996 sperm sorters worked as “standard-speed” systems, where the samples are sorted with not more than 0.84 kg/cm^2 of pressure giving a sort rate of about 350,000 spermatozoa/h (JOHNSON et al. 1989). New high-speed cell sorters like the MoFlo SX (Dakocytomation, USA) operate at sample pressures of up to 4.22 kg/cm^2, allowing to identify 30,000 events/sec and producing up to 15 million sorted spermatozoa of high purity above 90 % per hour. A major reason for this improvement is a modified nozzle that improved sperm orientation by more than 70 % (RENS et al. 1998). The nozzle was further refined by XY, Inc. (Fort Collins,

Colorado) to incorporate a ceramic nozzle tip (Cytonozzle^{TM}) and orientation of the sperm population now reaches up to 85 %.



Picture 2-1: Principals of Flow-cytometrically sperm sorting

Preparation and handling of semen before and after sorting has a major impact on semen quality and thereby fertility. Possible major sources for cell violation are the dye, laser light, the electric field and mechanical forces by high liquid pressure (MAXWELL and JOHNSON 1999). To minimize this damage the constant temperature, the osmolarity of buffers, pH and the sterility of the staining, the sheath, the collection and cryo-preservation media are essential (also see 2.6.).

Independent of the type of cell sorter, sample preparation begins with the staining of spermatozoa using Hoechst 33342 stain at 34°C - 38°C for 60-90 minutes. Labelled spermatozoa are introduced into the flowcytometer across the sample line under high pressure. Two streams of the sheath fluid with pressure just above the sample pressure surround the core stream in the injection tube. Oriented spermatozoa exit through the vibrating nozzle tip, which produces small droplets, theoretically containing one labelled spermatozoa only. Immediately after their exit, the laser light excites the fluorescent dye and the emitted light is collected through detectors in position 0° and 90°. The electric signals of the photocells are processed in a

computer. Cells failing to fulfil preset criteria are excluded from further analysis. The proper positioned cells are selected as X- or Y- chromosome bearing spermatozoa, according to the amount of emitted light from the flat sperm surface (0° detector). Based on this signal, the computer sends the signal to the wire loop to electrically charge the droplets accordingly. Charged droplets pass an electro-static field and are separately collected into tubes pre-filled with a collection extender. Sorted spermatozoa are then washed from the sheath fluid by centrifugation and the remaining sperm pellet is extended in a suitable medium. The composition of media differs in among species and also according to the following preservation process (HOLLINSHEAD et al. 2004; LINDSEY et al. 2002; RATH et al. 2003a; SEIDEL, JR. et al. 1996).

2.5 Reanalysis of sorted samples

Validation of sorted samples is performed immediately after sorting either by reanalysis in the sorter (WELCH and JOHNSON 1999), by fluorescence in situ hybridization (FISH) (KAWARASAKI et al. 1998) or by PCR (WELCH et al. 1995). Sort reanalysis for DNA has an advantage over FISH and PCR, since both techniques may take 3 to 4 h, whereas reanalysis in the flowcytometer requires less than 40 minutes. For a reanalysis in the sorter 100,000 spermatozoa are taken preferably from the originally sorted material or can be taken from an extra sort for reanalysis. Cells are sonicated to remove tails and Hoechst 33342 is added at a 10^{th} of the original concentration in order to maintain staining uniformity. Spermatozoa are not sorted but analyzed at very low speed in order to maximize orientation. DNA difference and histograms are analyzed by curve fitting to double Gaussian peaks (JOHNSON et al. 1987b) using the programs Summit to Gauss and Gaus7.

2.6 Effects of flowcytometrical sorting on the viability of spermatozoa

The exposure of highly specialized cell to flowcytometrical sorting may cause cell stress leading to visible and hidden sperm damages. Several studies have been performed after sorting determining effects on sperm viability and sperm fertilizing capacity. One of the most important questions in these studies was, whether the sorting technology damages sperm DNA, which could result in mutagenic changes of embryo development after fertilization. Exposure of somatic L1210 cells, labelled with

Hoechst 33342, to a UV-Laser indicated an occurrence of single-stranded DNA breaks, had cytotoxic effects on cell growth, blocked cells in G2-M phase and induced polyploidy (ERBA et al. 1988). Whether some of these changes may also occur during sperm sorting is not completely known so far, but several studies showed relatively low sensitivity of spermatozoa to changes of their DNA. It is proposed that highly condensed sperm DNA is more resistant against fluorescent stain (Hoechst 33342) and UV-Laser (MAXWELL et al. 2004) as compared to somatic cells. This has been confirmed by CATT et al. (1997) looking for nick translations and by SEIDEL and GARNER (2002), employing a chromatin stability assay, from which the investigators reported no negative effects. DNA damages may finally be indicated in embryo development and integrity of offspring. So far, no changes in embryo development have been reported (CRAN et al. 1993; HOLLINSHEAD et al. 2004; PROBST and RATH 2003; RATH et al. 1999). More than 40,000 offspring are born worldwide from sorted spermatozoa and in all studies progeny developed normal (MAXWELL et al. 2004). Profound studies of gestation length, birth weight, calving ease, calf vigour, weaning weight, abortion rate, and death rates (neonatal and through weaning) between animals inseminated with sorted semen and unsorted control, did also not reveal any significant difference (TUBMAN et al. 2004). Also PARILLA et al. (2004) did not find any effect neither on phenotypic abnormalities in offspring nor on sister chromatid exchanges nor on chromosome aberration frequencies in lymphocytes. However, it should be mentioned that in multiparous animals like the pig, litter size is reduced after insemination with sorted spermatozoa. This might not only be an effect of low sperm dosage per AI and sperm fertilizing ability, but may indicate a selective process during implantation (GROSSFELD et al. 2005).

Other sperm compartments are more sensitive to effects caused by sperm sorting. The percentage of capacitated spermatozoa increases (HOLLINSHEAD et al. 2003; KNÖPPEL 2001; MAXWELL et al. 1998), possibly due to a high dilution by the sheath fluid during sorting and consequently leading to a removal of protective substances present in seminal plasma (MAXWELL et al. 1998). On the contrary, the addition of seminal plasma to the collection fluid prevents capacitation or decapacitates spermatozoa (MAXWELL et al. 1996).

Motility of spermatozoa is also negatively affected by the sorting procedure, indicated after prolonged incubation in a thermo-resistant test (HOLLINSHEAD et al. 2003; RATH et al. 2003b). Some authors reported a negative correlation between working pressure of the flow system and motility. In consequence, most sperm sorters now use a lower working pressure of 30 to 40 psi than in the past (SUH and SCHENK 2003). Some indications are also available showing a direct correlation between sperm motility and the electric field, necessary to split both sperm populations. This is possibly caused by membrane polarisation of the sperm tail mid-piece and may temporarily disturb ATP synthesis. The dye Hoechst 33342 itself, used at concentrations for sperm sorting, did not show any negative effect on motility and pregnancy rates after artificial insemination with stained but unsorted boar spermatozoa (VAZQUEZ et al. 2002). The laser light was also correlated with inhibition of motility and immobilization of spermatozoa (MONTAG et al. 2000). It was found that exposure to laser light accelerates Ca^{2+} transport into irradiated bull spermatozoa (BREITBART et al. 1996), enhances Ca^{2+} binding to plasma membranes and inhibits Ca^{2+} uptake by mitochondria (LUBART et al. 1997).

An assessment of ram semen quality under semi-vivo conditions showed a shortage of the lifetime for sorted spermatozoa when compared to unsorted controls. Although similar numbers of unsorted and sorted frozen-thawed spermatozoa migrated through artificial cervical mucus after 1h, both displaying a similar pattern of binding to, and release from oviductal cell monolayer, sorted frozen-thawed spermatozoa were released more rapidly than unsorted spermatozoa (HOLLINSHEAD et al. 2003). These results indicate that sorted spermatozoa presumably have a shorter lifespan in the female genital tract than unsorted spermatozoa.

2.7 Livestock production with sex sorted spermatozoa

Since the first laboratory tests in the mid eighties, the flowcytometrical sexing technology has undergone major improvements up to a level that is now starting to be of interest for commercial application, at least in the bovine. Nevertheless, the output of sex-sorted spermatozoa is still limited compared to the number in a full ejaculate, as each single cell has to be identified and sorted. In consequence, strategies for insemination differ from normal AI and other biotechniques have to be

combined with sperm sorting. Table 1 shows the first reported production of offspring with flowcytometrically-sorted spermatozoa as seen in key literature.

Employing in vitro fertilization (IVF) or intracytoplasmatic sperm injection (ICSI) increases the efficiency of sperm sorting significantly. Depending on the species, 20 to 1000 sorted spermatozoa are required to fertilize an oocyte in an IVF system and theoretically one sperm cell is sufficient per oocyte when ICSI is applied (PROBST and RATH 2003). In different species offspring were born after in vitro fertilization with sorted spermatozoa. CRAN et al. (1993) reported birth of first calves after in vitro fertilization and in the same year porcine blastocysts were produced from IVF (RATH et al. 1993) Four years later, first piglets were born following in vitro fertilization with sorted spermatozoa, employing in vivo matured porcine oocyte (RATH et al. 1997). This procedure was successfully repeated with in vitro matured oocytes (ABEYDEERA et al. 1998). LU et al. (1999) reported successful use of fresh or frozen sorted bovine spermatozoa for IVF, but in some experimental groups a decrease in blastocyst formation occurred compared to controls. IVF with sex sorted frozen/thawed spermatozoa resulted in lambs after embryo transfer in adult (HOLLINSHEAD et al. 2004) and prepuberal recipients (MORTON et al. 2004).

Table 2-1: Methods of fertilization with sex sorted spermatozoa in different farm animal species.

Kind of flowcytometer	Species	Semen preservation	Fertilization method	References
Standard	Cattle	fresh	Conventional AI	(MORRELL et al. 1988)
Standard	Cattle	fresh	IVP(fresh)/ET	(CRAN et al. 1993)
Standard	Cattle	fresh	IVP (F/T)/ET	(CRAN et al. 1994)
Standard	Cattle	fresh	DIU-AI	(SEIDEL, JR. et al. 1997)
High speed	Cattle	fresh and frozen	Conventional AI and DIU-AI	(DOYLE et al. 1999; SEIDEL, JR. et al. 1999)
High speed	Horse	fresh	DIU-AI	(BUCHANAN et al. 2000)
High speed	Horse	fresh and frozen	DIU-AI (Hysteroscopy)	(LINDSEY et al. 2002)
Standard	Human	fresh	IVP/ET	(LEVINSON et al. 1995)
Standard	Human	fresh and frozen	AI, ICSI, IVF	(FUGGER 1999)
Standard	Rabbit	fresh	Surgical AI uterus	(JOHNSON et al. 1989)
Standard	Sheep	fresh	ICSI/ET	(CATT et al. 1996)
Standard	Sheep	fresh	Laparoscopic AI	(CRAN et al. 1997)
High speed	Sheep		Laparoscopic AI and DIU-AI	(HOLLINSHEAD et al. 2001)
Standard	Swine	fresh	Surgical AI oviduct	(JOHNSON 1991)
Standard	Swine	fresh	IVF/ET	(RATH et al. 1997)
High speed	Swine	frozen	Surgical AI oviduct	(JOHNSON et al. 2000)
High speed	Swine	fresh	ICSI	(PROBST and RATH 2003)
High speed	Swine	fresh	DIU-AI	(RATH et al. 2003a; VAZQUEZ et al. 2003)

IVF: In vitro fertilization; IVP: In vitro production including in vitro maturation, in vitro fertilization and in vitro culture; AI: Artificial insemination; DIU-AI: Deep intra-uterine insemination; F/T: frozen/thawed; ICSI: Intra-cytoplasmatic sperm injection; ET: Embryo Transfer

The main instrument to produce progeny with sexed spermatozoa is of course artificial insemination. As sperm numbers for AI differ among species, and sorting rates are species independent, different modifications have to be made to optimize the use of sorted spermatozoa. In the bovine, inseminations with about 2 millions sexed frozen/thawed spermatozoa have been reported to be successful. However, in highly diluted sperm samples, male specific effects occur and before use of a specific bull, semen has to be pre-tested, before acceptable fertility can be expected (SEIDEL, JR. et al. 1996).

In other domestic animal species it is not possible to achieve normal pregnancy rates after drastically reduction of spermatozoa with standard insemination techniques. Different alternatives were developed to overcome this problem, for example employing surgical insemination into oviduct or uterus. JOHNSON et al. (1989) reported the birth of first rabbit litters that were born after surgical insemination into both uterine horns with only $3x10^5$ sex sorted spermatozoa. This procedure was successfully repeated also in swine (JOHNSON 1991) and sheep (CRAN et al. 1997). However, surgical insemination is limited to research, since it demands a well-trained surgeon, special equipment and underlies at least in Germany animal welfare legislation.

Recently, a flexible catheter for porcine non-surgical deep intrauterine insemination was developed and allows to reduce the amount of spermatozoa. Successful pregnancies and birth of life piglets with flowcytometrically sorted spermatozoa was reported after AI with $50x10^6$ sex sorted (RATH et al. 2003a) and $70x10^6$ bold sorted spermatozoa (VAZQUEZ et al. 2003). Also in mares pregnancies were achieved after hysteroscopic insemination onto the utero-tubal papilla with $5x10^6$ sex sorted spermatozoa (LINDSEY et al. 2002). In cattle and as recently reported also in Water Buffalo (PRESICCE et al. 2005) acceptable pregnancies rates can be achieved after artificial insemination with $2x10^6$ sex sorted spermatozoa, using a normal insemination device. Insemination with liquid sorted semen has been applied in heifers, but proved to be only successful in the first few hours after sorting (SEIDEL, JR. et al. 1996; SEIDEL, JR. et al. 1997). Time depended reduction of fertilizing capacity of sorted spermatozoa can be avoided using frozen/thawed spermatozoa (SCHENK et al. 1999). Although frozen/thawed sex sorted spermatozoa have been

used to inseminate heifers, pregnancy rates were in general lower compared to unsorted control semen (SEIDEL, JR. et al. 1999).

The ability to achieve acceptable pregnancy rates with a low number of spermatozoa depends on individual potentials of a bull and directly affects the fertilizing capacity of sex sorted spermatozoa (FLINT et al. 2003). Semen deficiencies can be categorized as compensable or uncompensable. Seminal differences among males in numbers of sperm required to reach maximum pregnancy rates are considered to be compensable. Once the maximum pregnancy rate of a male has been reached, further spermatozoa are inseminated without effect. Differences in pregnancy rates due to the male are considered to be uncompensable (SAACKE et al. 2000). Different studies indicated the significance of bull effects that are already recognizable at a dosage of $15x10^6$ spermatozoa (SENGER et al. 1984). Better fertility status and embryo quality was found with increased sperm number from $20x10^6$ to $100x10^6$ (NADIR et al. 1993). The selection of bulls for low dose insemination with only $2x10^6$ sex sorted spermatozoa is therefore important. Heterospermic insemination was shown as a powerful tool to identify low and high fertility bulls after flowcytometrical sorting (FLINT et al. 2003).

Another possible approach for selection of bulls with high fertilizing capacity is the estimation of accessory spermatozoa trapped in the zona pellucida of an embryo. DEJARNETTE et al. (1992) reported positive correlations between the number of accessory spermatozoa per embryo, fertilization rate, and embryo quality.

Timing of insemination is also an important factor influencing fertility. DALTON et al. (2000) reported increased fertilization rates and a higher number of recovered embryos with accessory spermatozoa in superovulated, nonlactating Holstein cows, inseminated 24 h after onset of estrus. Positive effects on accessory sperm number after insemination in the late estrus was reported by SAACKE et al. (2000).

Effects on pregnancy rates caused by females were reported in several studies. SARTORI et al. (2002) reported a higher fertilization rate, superior quality scores and more nuclei per embryo in heifers compared to lactating cows and superior quality of embryos in dry cows compared to lactating cows. Higher conception rates were also detected in primiparous as compared to older cows (TENHAGEN et al. 2004). Sorted

spermatozoa are because of higher fertility preferably used for AI in heifers (SEIDEL, JR. 2003).

The use of flowcytometrically sorted bull spermatozoa has reached its final steps before broad commercial application. An economically satisfactory utilization of sorted semen depends mainly on cost/benefit calculations. In the USA, the retail premium for one dose of sexed semen ranges from 10 to 50 $ above the price of an average dose of unsexed semen. SEIDEL, JR (2003) made an extended calculation for commercial application of sex sorted spermatozoa. He investigated the effect of pregnancy rate and difference in the price between male and female calves on the break-even costs after application of sex sorted spermatozoa with relatively high fertilizing capacity (10 % decrease compared to unsorted semen). The highest effect was observed for the difference in the price between male and female calves. In herds with high pregnancy rates (60 % for unsorted and 54 % for sorted semen) the break-even value for extra costs per dose of sorted semen would be 7.11, 25.16 and 43.52 $, if the difference between male and female calves was estimated at 180, 280 and 380 $, respectively. The break-even value for extra costs per dose sorted semen would be 1.27, 13.26, 25.46 $, for the same difference in price between male and female calves, in herds with low pregnancy rates (40% for unsorted and 36% for sorted semen). This data shows that commercial application of sex sorted spermatozoa depends on several factors, but the most important factor remains an acceptable fertilizing capability of sex-sorted spermatozoa. Therefore, further research is required to increase the fertilizing capacity of sex-sorted spermatozoa.

3 Chapter 1: Gender pre-selection of X- and Y- chromosome bearing spermatozoa and effects of processing on sperm quality and fertility

3.1 Abstract

The objectives of the study were to analyse the effects of semen processing after flow-cytometrical sorting and subsequent freezing in liquid nitrogen on the quality and fertilizing capacity of frozen/thawed spermatozoa. Quality of the frozen/thawed semen was evaluated by motility estimation, morphology analysis, membrane stability (6-CFDA/PI) test, capacitation test (FITC-PNA/PI staining with addition of L-α-Lysophosphatidylcholine) and fertility assessment in an insemination trial on a farm. Prolonged high dilution of flowcytometrically sorted spermatozoa before freezing had a significant negative effect on motility ($P<0.001$) and capacitation status ($P<0.05$). Positive effects on membrane stability were seen when glycerol was added at 5°C shortly before straws were frozen compared to a system where glycerol was added before the equilibration process at room temperature ($P<0.001$). Independently of sperm processing after sorting, sexed spermatozoa had significantly more damaged acrosomes and morphological abnormalities ($P<0.001$). In addition sex sorted frozen/thawed spermatozoa (immediate centrifugation and glycerol addition at 5°C) and unsorted frozen/thawed semen samples were submitted to a thermo-resistance test at 37°C. Immediately after thawing no significant difference was seen in the percentage of motile spermatozoa between sorted and unsorted semen samples. However, after further incubation at 37°C, motility of sorted spermatozoa was significantly lower compared to unsorted spermatozoa after 3 h ($P<0.001$) and 6 h ($P<0.05$). The pregnancy rates after insemination with sex-sorted and unsorted spermatozoa from same ejaculates was tested in a field trial in heifers and cows with natural and synchronised oestrus cycles. In natural oestrus, more animals became pregnant after artificial insemination with unsorted than with sex-sorted spermatozoa (56.5 % vs. 17.6 %; $P<0.001$) No significant differences were observed between unsorted and sex-sorted frozen/thawed semen samples after artificial insemination of the animals with synchronised oestrus (36.4 % vs. 21.3 %; $P=0.076$).

3.2 Introduction

Flowcytometrically sorted frozen/thawed spermatozoa have been successfully applied to artificial insemination in cattle (MAXWELL et al. 2004). However, semen quality and pregnancy rates after artificial insemination were rather low compared to unsorted frozen/thawed semen (SEIDEL, JR. et al. 1999; SEIDEL, JR. et al. 1996; SEIDEL, JR. et al. 1997). Main sources that may reduce the semen quality during flowcytomerical sorting are:

1. UV-Laser (LUBART et al. 1997; MONTAG et al. 2000),
2. Pressure (SUH and SCHENK 2003),
3. Dilution effects (MAXWELL et al. 1996) and
4. Electric charge/electro-static field.

The dye itself at concentrations used for sorting does not have a significant impact on fertility of boar semen (VAZQUEZ et al. 2002). Exposure to laser light accelerated Ca^{2+} transport into irradiated bull spermatozoa (BREITBART et al. 1996), enhanced Ca^{2+} binding to plasma membranes and inhibited Ca^{2+} uptake by mitochondria (LUBART et al. 1997). Because of this insults the processing of sorted spermatozoa before freezing needs to be adjusted in order to obtain high quality and fertility rates after insemination.

Additionally, spermatozoa are highly diluted during sorting ($8x10^5$ spermatozoa/1 mL) by the sheath fluid and the sperm concentration in the collection tube is approximately one million spermatozoa/mL. High dilution was found to be detrimental for sperm motility and viability and could also affect the fertilizing capacity of spermatozoa (MAXWELL et al. 1998; MAXWELL and JOHNSON 1999). For further processing, sexed sperm samples have to be centrifuged. Centrifugation however, was found to increase the production of reactive oxygen species, which are detrimental for the sperm viability (SHEKARRIZ et al., 1995). As sorted spermatozoa are sensitive to membrane insults that may occur during centrifugation and storage at room temperatures, one objective of the present study was to test whether it is better to keep the highly diluted sorted spermatozoa at room temperature for a few hours and centrifuge all sorted samples together after 4 to 6 hours or to perform centrifugation immediately after sorting, chilling and freeze each sample separately.

Centrifuged spermatozoa need to be diluted in a suitable extender, cooled to 5°C and frozen in liquid nitrogen. The semen extender has to provide a suitable environment for survival and maintaining the viability of spermatozoa. The composition of semen extenders is mainly based on an energy resource (sugars such as glucose, fructose, lactose) and a buffer medium of different inorganic or organic salts. Additionally, the semen extenders contain components such as egg yolk, skim milk, specific amino acid, glycerol, detergent and antioxidants, which protect the spermatozoa (PICKETT and AMANN 1993). Egg yolk is an important component of semen extenders. The protective action of egg yolk is assumed to be due to membrane protection by low density lipoproteines (MOUSSA et al. 2002), and the antioxidant ability of the phosphoprotein Phosvitin, which serves as an iron-carrier and protects spermatozoa against the Fenton reaction (ISHIKAWA et al. 2004). Recently, egg-yolk of freezing extender for bull semen has been successfully replaced with soybean extract (THUN et al. 2002), but Tris egg-yolk freezing extenders are still preferred for freezing sorted bull semen (SCHENK et al. 1999). Glycerol is a cryoprotector, which is toxic to spermatozoa. Its toxicity is temperature dependent as seen especially in human semen (CRITSER et al. 1988). Therefore, some authors suggest the addition of glycerol after cooling the semen to 5°C (PICKETT AND AMANN 1993), whereas others prefer room temperature (VIDAMENT et al. 2000; VOLKMAN and VAN ZYL 1987). Therefore the second goal of our research was to determine the effect of glycerol addition at different temperatures.

3.3 Material and Methods

3.3.1 Processing of the semen

Semen was collected from two fertility proven Holstein Friesian bulls with an artificial vagina and kept in a water bath at 27°C. Motility was estimated under phase contrast microscope at 37°C and only ejaculates with >70 % total motility were used in the experiment. Concentration of ejaculated spermatozoa was determined with a Thoma counting Chamber (Thoma neu®, Hecht, Sontheim, Germany). One part of the raw semen was diluted with Tris-sample fluid to $1x10^8$ spermatozoa per millilitre. Diluted samples were labelled with 15, 20 and 25 µL of 8.12 mM Hoechst 33342 solution and incubated at 37°C for 1.5 hours. The labelled samples were pre-tested with a flowcytometer and the concentration of Hoechst 33342 stain giving the best

resolution of the two sperm populations was used for daily sort. Labelled and incubated semen samples were kept at 22°C in the dark and sorted within 7 hours after onset of incubation. The remaining unlabelled part of the ejaculates was frozen according to a standard protocol described by THUN et al. (2002). All semen samples were gradually diluted with the same amount of TRIS egg-yolk freezing extender I and II, giving 6.4% final concentration of glycerol. Extender II was added to the semen, depending on the experimental design, at room temperature or at 5°C. Final sperm concentration was set to $13.2x10^6$ spermatozoa/ml or $3.3x10^6$ spermatozoa/straw. Diluted semen was then filled into 0.25 mL straws (Minitüb, Tiefenbach, Germany), cooled to 5°C within 2 h and frozen in liquid nitrogen. Freezing of the samples was performed in closed Styrofoam box (30 cm x 40 cm x 85 cm = high x bright x length). Briefly, straws were placed on metal holder in nitrogen vapour 3-5 cm above LN_2 for 15 minutes. Frozen samples were than plunged into LN_2 and kept in the semen container (in LN_2) until used for analysis or artificial insemination.

3.3.2 Sperm Sorting

Sperm sorting was performed according to the Beltsville Sperm Sorting Technology (JOHNSON et al. 1999). Semen samples, prepared as described above, were filtered through a 51 µm Cell Strainer grid (Falcon Becton Dickinson and Company, Franklin Lakes, NY, USA) and then supplemented with 1 µL food dye solution FD&C#40 (Warner Jekinson Company Inc. St. Louis, MO USA). Sorting was performed with a high speed flowcytometer MoFlo SX, (Dakocytomation Fort Collins, CO, USA, equipped with an argon UV-Laser (Coherent Laser®, Inova I 909-6, Dieburg, Germany), set to 200 mW output. Samples were sorted in the presence of Tris-sheath fluid at an average event rate of 25000 cells/sec giving a sorting rate of 3300 cells/sec. Spermatozoa were collected into 10 mL conical plastic tubes (Greiner, Nürtingen, Germany) pre-filled with 500 µL TEST-yolk extender (JOHNSON 1991). After collection of 8 millions sorted spermatozoa in each collection tube, samples were centrifuged at 840xg for 20 minutes. The supernatant was discharged and the sperm pellet was resuspended with TRIS egg-yolk freezing extender I and II, then filled into straws and frozen in liquid nitrogen. Time of centrifugation and the temperature of glycerol addition were adjusted according to the experimental design.

3.3.3 Experimental design

Tubes containing sorted spermatozoa were submitted to three different protocols. Sorted sperm samples of the first group (group A) were kept at room temperature until the end of daily sorting (4-6 hours after onset of sorting). Centrifugation was performed at the end of this holding period. The sperm pellet was gradually diluted with TRIS freezing extender I and II to a concentration of $20.5x10^6$ spermatozoa/mL. Plastic straws (Fine pailette, 0.25 mL, Minitüb, Tiefenbach, Germany) were filled with 3.3 millions spermatozoa and cooled to 5°C over a 2 hours period. Freezing in the vapour of LN_2 was performed approx. 4 hours after glycerol addition (Table 1).

Sorted semen samples in the second group (group B) were processed similarly to samples of group A, except that centrifugation was performed immediately after collection tubes were filled with $8x10^6$ spermatozoa. The difference between group B and C was the addition of TRIS freezing extender II at 5°C, 45 minutes before freezing in LN_2 (Table 1). Control samples of unsorted semen were frozen as described for group A.

Table 3-1: Processing of the sorted spermatozoa

	Centrifugation	Glycerol addition
Group A	4-6 h after onset of sorting	Room temperature
Group B	Immediately after sorting	Room temperature
Group C	Immediately after sorting	5°C
Control	Unsorted	Room temperature

All sorted semen samples and unsorted controls from one sorting day were frozen at the same time according to the protocol described under 3.3.1. Frozen samples were kept in LN_2 and analysed or used for insemination within 2-5 months after freezing. The straws were thawed at 37°C for 17 seconds.

3.3.4 Analysis of frozen thawed samples

3.4.4.1 Reanalysis of sorting purity

Reanalysis to identify sort purity was performed after thawing. Aliquots of 1 million spermatozoa were taken and diluted to 1mL with TRIS-sample fluid, supplemented with 20 µl of a 0.8 mM solution of Hoechst 33342 and incubated for 20 minutes at 37°C. Thereafter, samples were sonicated and filtered as described before (WELCH and JOHNSON 1999). Reanalysis was performed at 60 events/sec. The analysis of purity was performed by a curve fitting model.

3.3.4.2 Motility analysis

Motility of raw semen samples as well as frozen/thawed semen samples was analysed under a phase-contrast microscope (Olympus BX 60, Hamburg, Germany) equipped with heating plate at 37°C. Two drops and at least three fields per drop were analysed in each sample at 100x magnification.

3.3.4.3 Analysis of morphological sperm abnormalities

Morphological abnormalities (MAS) and acrosome integrity were analysed under a phase-contrast microscope (Olympus BX 60, Hamburg, Germany) at 1000x magnification after fixation in Hancock solution. At least 200 spermatozoa were examined per sample. Abnormalities of spermatozoa were divided into damaged acrosomes and morphological abnormal spermatozoa. All Classification parameter are summarized in appendix (table 12-1).

3.3.4.4 Viability and membrane stability of spermatozoa (CFDA/PI)

Samples were prepared by mixing 3.3 µL CFDA (*51.04 mM 6-Carboxyfluorescein diacetate diluted in Dimethyl sulfoxide*) and 6.6 µL propidium iodide solution (PI) (*mixture of 200 µL of 7.48 mM 3,8-Diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide and 400 µL fixative solution: 68 µL of a 37 % formaldehyde solution per 10 mL distilled water*) with 10 µL semen sample. Samples were incubated at room temperature in darkness for 10 minutes. From each sample two drops and at least 200 spermatozoa were analysed under a fluorescence

microscope (Olympus BX 60; U-MNIB filter, Hamburg, Germany) at 400 x magnification. Spermatozoa were divided into two groups: viable spermatozoa with intact plasma membrane (CFDA positive and PI negative) and morbid spermatozoa (CFDA positive and PI positive).

3.3.4.5 Capacitation status of the spermatozoa (FITC-PNA/PI; LPC)

Capacitation status of spermatozoa was assessed with a modified protocol as described by MCNUTT and KILLIAN (1991). One Eppendorf cup was filled with 1 mL TRIS-sample fluid and another with 800 µL TRIS-sample fluid and 200 µL LPC solution (*500µg L-α-Lysophosphatidylcholine and 1 mL Tris-sample fluid*). Tubes were equilibrated for 30 minutes at 39°C, than supplemented with 50 µL of semen and incubated for another 10 minutes at 39°C. Centrifugation of both tubes was then performed at room temperature at 500xg for 5 minutes. Supernatant was removed to 100 µL and mixed with the sperm pellet, supplemented with 2 µL FITC-PNA (*2 mg FITC-PNA in 2 mL PBS*) and 4 µL PI solution (*1.50 mM 3,8-Diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide and 154 mM NaCl in bi-distillated water*) and incubated another 5 minutes at 39°C. The samples were then supplemented with 10 µL paraformaldehyde (*1 % in PBS*) and analysed immediately. At least 200 spermatozoa in two drops were examined under fluorescence and phase contrast microscope (Olympus BX 60; U-MNIB filter) at 400 x magnification. Spermatozoa were divided into three groups: viable (PNA-negative, PI-negative), acrosome reacted (PNA-positive) and membrane damaged with intact acrosomes (PNA-negative, PI-positive). Parameters are summarised in appendix (table 12-2). Percentage of capacitated spermatozoa was calculated from the difference between the percentage of acrosome reacted spermatozoa before and after addition of LPC.

3.3.5 Artificial insemination

Sorted frozen/thawed spermatozoa of groups A and B were used only for laboratory assessment, whereas spermatozoa of group C and unsorted controls were used also for artificial insemination. Heifers and cows were submitted to routine insemination on one farm and were divided into two groups. Animals in the first group were inseminated into the uterine body 12-24 hours after onset of natural oestrus. Cows

and heifers of the second group were synchronised with GnRH, and PGF-2α (TENHAGEN et al. 2003). In detail, cows received 100 µg GnRH (Depherelin Gonavet Veyx®, Schwarzenborn, Germany) at day -10, further 0.5 mg Cloprostenol (PGF-2α analogue, Essex, München, Germany) at day -3 and again 100 µg GnRH at day -1. Timed artificial inseminations into uterine body were performed 24 h after the second dose of GnRH on day 0. Heifers were synchronised with single injection of 0.5 mg Cloprostenol and animals coming into heat were inseminated 72 hours later into uterine body.

Pregnancies were diagnosed 30-60 days after insemination by transrectal ultrasonography (Aloka®; 5 MHz). All pregnant animals were allowed to go to term.

3.3.6 Statistical analyses

Data were analysed with the statistic programme “SigmaStat 2.03”. Descriptive statistic was used for analyses of mean and standard deviation. Laboratory results were tested for normal distribution and then analysed by One-way ANOVA or ANOVA on Ranks and Tukey Test. Fertility results were tested with Chi-square test. Statistical difference between groups were accepted to be significant at $P<0.05$ %. Results are presented as mean ± standard deviation.

3.4 Results

Frozen/thawed semen samples were analysed after incubation at 37°C for 15 minutes (Table 3-2). Percentage of motile spermatozoa was significantly lower in group A compared to groups B, C and control samples. Significantly higher percentage of motile spermatozoa was observed for bull 2 in comparison to bull 1 in unsorted control samples ($P= 0.012$).

Table 3-2: Total motility of spermatozoa (%) after thawing

n=12	Group A	Group B	Group C	Control
Bull 1	$16,7 \pm 14,5^{a}$	$55,0 \pm 8,4$	$65,0 \pm 3,0^{b}$	$60,8 \pm 6,6^{b}$
Bull 2	$32,9 \pm 22,9^{a}$	$57,5 \pm 7,6$	$64,6 \pm 5,4^{b}$	$70,0 \pm 3,2^{b}$
Together	24.8 ± 20.5^{a}	56.3 ± 7.7^{b}	64.8 ± 4.3^{b}	65.4 ± 6.9^{b}

Values with different superscripts differ significantly ($P<0.05$).

The semen samples from group C, which showed to be superior to other two groups of sorted spermatozoa and unsorted control samples were thawed separately and subjected to thermo-resistance test at 37°C. Analysis of motility in frozen/thawed samples, after incubation on 37°C for 15 minutes did also not reveal any significant difference between sorted samples and unsorted controls. After further incubation at 37°C, the motility decreased faster in sorted samples and was significantly higher in control samples after 3 and 6 hours of incubation (Figure 1).

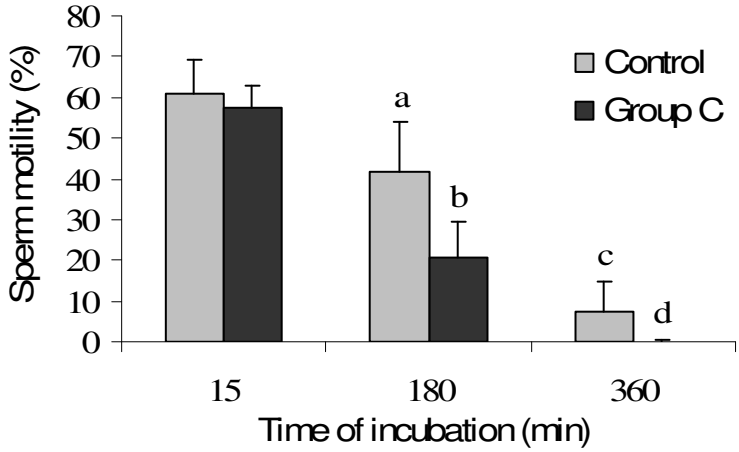


Figure 3-1: Motility of spermatozoa in frozen/thawed semen samples during incubation over a 6 h (360 min) period at 37°C (n= 12); Superscripts a and b differ significantly (P<0.001); c and d differ at P<0.05.

Significantly higher percentages of spermatozoa with damaged acrosomes were observed in all groups of sorted spermatozoa compared to unsorted control (P<0.001). The difference between the groups of sorted spermatozoa was not statistical significant. A significant difference between sorted semen and unsorted controls was also observed for the MAS (P<0.001).

Table 3-3: Percentage of spermatozoa with damaged acrosomes and morphologically abnormal acrosomes (MAS)

(n= 12)	Group A (%)	Group B (%)	Group C (%)	Control (%)
Acrosomes	32.6 ± 7.0[a]	32.0 ± 7.6[a]	29.2 ± 5.3[a]	17.9 ± 7.3[b]
MAS	43.3 ± 7.8[a]	44.0 ± 8.1[a]	37.4 ± 6.8[a]	28.6 ± 7.0[b]

Values with different superscripts differ significantly (P<0.001).

Bull 1 had compared to bull 2 statistically significant higher percentages of damaged acrosomes in group A (P<0.001) and B (P=0.041) and a significantly higher percentage of MAS in group A (P= 0.009). As presented in table 3-4, bull 1 had significantly higher percentage of damaged acrosomes and MAS in all groups of sorted spermatozoa compared to unsorted semen. For bull 2 this differences were not statistically significant.

Table 3-4: Difference in the percentage of damaged acrosomes and morphological abnormal spermatozoa (MAS) between bull 1 (B1) and bull 2 (B2)

(n=6)	Group A (%)	Group B (%)	Group C (%)	Control (%)
Acrosomes (B1)	37.2 ± 5.2[a]	36.3 ± 7.9[a]	29.9 ± 3.7[a]	14.2 ± 5.5[b]
Acrosomes (B2)	28.1 ± 5.7	27.7 ± 4.4	28.4 ± 6.6	21.7 ± 7.4
MAS (B1)	47.3 ± 6.0[a,e]	47.3 ± 8.4[a,e]	38.7 ± 5.7[c,f]	26.0 ± 4.8[b,d]
MAS (B2)	39.3 ± 7.6	40.7 ± 6.9	36.2 ± 7.6	31.2 ± 8.3

Values with superscripts differed significantly a:b (P<0.001); c:d and e:f (P<0.05).

Percentage of viable (CFDA-positive) spermatozoa was significantly higher (P<0.001) in group C (45.7 ± 6.3 %) compared to group A (28.3 ± 4.9 %), B (30.6 ± 8.1%) and control samples (33.5 ± 6.7 %). Percentage of morbid spermatozoa (CFDA and PI positive) was significantly lower (P<0.001) in group C (36.5 ± 5.8 %) and control (35.8 ± 6.3 %) compared to group A (51.4 ± 7.1 %) and B (46.0 ± 8.3 %). Bull 1 had compared to bull 2 significantly (P<0.05) lower percentage of viable spermatozoa in all tested groups and significantly (P<0.05) higher percentage of

morbid spermatozoa in group C. As presented in table 3-5, both bulls had significantly higher percentages of viable spermatozoa in group C compared to group A, B and control samples. Both bulls had significantly higher percentages of morbid spermatozoa in group A compared to group C and control samples. Bull 2 had also a significantly higher percentage of morbid spermatozoa in group A compared to group B and in group B compared to group C.

Table 3-5: Difference in the percentage of viable (CFDA+) and morbid (CFDA+/PI+) spermatozoa between bull 1 (B1) and bull 2 (B2)

(n=6)	Group A (%)	Group B (%)	Group C (%)	Control (%)
CFDA+ (B1)	26.0 ± 4.7[a]	25.3 ± 4.7[a]	43.0 ± 4.7[b]	28.7 ± 4.2[a]
CFDA+ (B2)	30.6 ± 4.0[a]	35.9 ± 7.4[a]	48.3 ± 6.7[b,c]	38.2 ± 5.1[d]
CFDA+/PI+ (B1)	50.8 ± 8.7[c]	48.8 ± 9.3	40.0 ± 3.7b[d]	37.0 ± 7.4[d]
CFDA+/PI+ (B2)	52.0 ± 5.6[a,c]	43.2 ± 7.0[d,e]	33.3 ± 5.6[b,f]	34.6 ± 5.5[b]

Values with superscripts differed significantly a:b (P<0.001); c:d and e:f (P<0.05).

A significant higher percentage of capacitated spermatozoa was observed in group A in comparison to both other sorted groups and control. Bull 1 had in comparison to bull 2 statistical significant lower percentages of capacitated spermatozoa in group B (9.0 ± 3.6 % and 13.8 ± 3.4 %, respectively) and C (9.1 ± 2.6 % and 13.6 ± 5.0 %, respectively).

Table 3-6: Percentage of capacitated spermatozoa (PNA/LPC-Test)

Group A (%)	Group B (%)	Group C (%)	Control (%)
16.3 ± 4.0[a]	11.4 ± 4.2[b]	11.3 ± 4.5[b]	7.9 ± 4.3[b]

(n= 12) Values with different superscripts differ significantly (P<0.05).

Significantly more pregnant animals were observed after AI with unsorted semen compared to groups with flowcytometrically sorted semen. Significantly more

pregnancies were achieved in bull 2 compared to bull 1 for the sorted semen (P<0.005) and in cows compared to heifers for the unsorted controls (P<0.007).

Table 3-7: Pregnancy rates and number of animals inseminated in the natural oestrus with flowcytometrically sorted spermatozoa and unsorted controls

	Sorted spermatozoa		Controls	
	(%)	n	(%)	n
Bull 1	12.1[a]	66	57.0[b]	128
Bull 2	26.2[c]	42	55.7[d]	79
Cows	18.8[a]	69	47.3[b]	110
Heifers	15.4[a]	39	67.0[b]	97
total	17.6[a]	108	56.5[b]	207

Values with superscripts differed significantly a:b (P<0.001); c:d (P<0.05).

Insemination with unsorted semen in animals with synchronized oestrus led to significantly lower pregnancy rates compared to animals inseminated in natural oestrus (P= 0.012). No difference was observed in animals inseminated with sorted spermatozoa in synchronised and normal oestrus (P= 0.629). Overall pregnancy rates in the synchronised animals did not differ between sorted spermatozoa and unsorted controls (P= 0.076). Significantly more pregnancies were observed for cows and bull 1 after insemination with unsorted compared to sex sorted spermatozoa. Sex ratios were as predicted by resort analysis different from the 50:50 ratio (P<0.001), and 84.5 % of the born offspring were females.

Table 3-8: Pregnancy rates and number of animals inseminated in the synchronised oestrus with flowcytometrically sorted semen and unsorted controls.

	Sorted spermatozoa		Controls	
	(%)	n	(%)	n
Bull 1	12.2[a]	49	38.5[b]	26
Bull 2	32.5	40	34.5	29
Cows	16.9[c]	65	35.7[d]	42
Heifers	33.3	24	38.5	13
total	21.3	89	36.4	55

Values with superscripts differed significantly a:b (P<0.001); c:d (P<0.05).

3.5 Discussion

The goal of this study was to analyse the effects of different modifications in sperm processing after sorting on the quality of frozen/thawed spermatozoa and to investigate the differences in the quality and fertility between sorted and unsorted frozen/thawed semen. Different processing of sorted spermatozoa had a significant impact on the quality of the frozen/thawed spermatozoa. Post thaw motility of spermatozoa was significantly higher in group B, C and unsorted controls compared to the samples in group A. Further incubation at 37°C showed significant reduction of motility in sorted samples compared to controls. This may reflect the situation in the female genital tract and sorted spermatozoa that have been processed especially according to group C have little lifetime after AI to reach the oocyte in the oviduct. Therefore, it has been proposed to inseminate closer to the expected time of ovulation and to inseminate deep into the uterine horn in order to avoid sperm losses during the transport through the uterus and to shorten the time interval between insemination and the passage through the utero-tubal junction.

A reason for the lower quality and the shorter lifespan could be the sorting itself (SUH and SCHENK 2003). However, as the sorting was identical for groups A, B and C, it is likely that the post-sorting process has a major impact on semen quality. The observed reduction of motility in group C may have been caused by reactive oxygen species (ROS) production. Exposure of spermatozoa to higher temperatures and centrifugation are known to increase the production of ROS and consequently lipid peroxidation (ALVAREZ and STOREY 1985; SHEKARRIZ et al. 1995). Removal of seminal plasma by high dilution during sorting promotes the lower resistance of sorted spermatozoa against such damages (BARRIOS et al. 2000; GARNER et al. 2001; MAXWELL et al. 1998). Increased morphological damages especially of acrosomes may also indicate a higher ROS production (ICHIKAWA et al. 1999). The percentage of capacitated spermatozoa was calculated from the difference between acrosome reacted spermatozoa before and after LPC treatment. A statistically higher percentage of capacitated spermatozoa was observed in group A in comparison with unsorted control samples. One of the reasons for capacitation and acrosome reaction is the lipid peroxidation of the sperm plasma membrane (ICHIKAWA et al. 1999; KIM and PARTHASARATHY 1998; OEHNINGER et al. 1995). The results indicate that

immediate centrifugation and replacement of sorting extender with an extender containing egg yolk, which has an antioxidant ability (ISHIKAWA et al. 2004; LU and BAKER 1986), offered protection against ROS.

Differences between groups B and C are mainly caused by the presence of Glycerol at room temperature. It is well known that Glycerol is toxic to spermatozoa and therefore should be added to sorted semen shortly before the freezing process starts (CRITSER et al. 1988; GIL et al. 2003).

Labelling of spermatozoa with CFDA and PI resulted in three distinct populations: green (enzyme active with intact plasma membrane), green/red (enzyme active with damaged plasma membrane), red (dead) (GARNER et al. 1986; HARRISON and VICKERS 1990; SÖNDERQUIST L. et al. 1997). The most important information in this test is the percentage of viable and membrane intact spermatozoa, represented in the green population. The highest percentages of viable and membrane intact spermatozoa were detected in group C. In groups A and B significantly more cells were damaged; it is also interesting that control samples were more damaged than the sorted sperm of group C. The reason for this result is the addition of food dye to the spermatozoa before sorting. Food dye only enters into spermatozoa with damaged plasma membranes, reduces the emission of Hoechst 33342 stain and enables their exclusion during sorting (SCHENK et al. 1999).

A statistical significant effect of the bull was found for motility, morphological changes, viability and the percentage of capacitated spermatozoa. These results are in agreement with other studies showing significant bull effect on the quality and the fertilizing capacity of spermatozoa, especially if the spermatozoa are highly diluted (JANUSKAUSKAS et al. 1996; SENGER et al. 1984). Further, they also indicate the importance of bull selection for success of flow-cytometrical sorting (FLINT et al. 2003).

Sperm fertilizing abilities were further tested in the field insemination trial. Pregnancy rates after AI during natural oestrus were lower for sorted spermatozoa (17.6 %) as compared to unsorted controls (56.5 %) and significantly affected by the bull ($P<0.005$). In synchronised animals the difference in pregnancy rates between sorted

and unsorted semen dropped to 15 % and pregnancy rates for bull 2 did not differ between sorted samples and unsorted controls.

This results showed a significant effect of the bull on the pregnancy rates after artificial insemination with sex sorted spermatozoa, which is in agreement with other reports indicating high importance of bull selection for insemination with reduced number of spermatozoa (FLINT et al. 2003; JANUSKAUSKAS et al. 1996; SENGER et al. 1984). Since the differences between the bulls were observed only for the sex sorted and not for the unsorted semen samples, the results also indicate the variability in resistance of spermatozoa to flow-cytometrical sorting between the semen donors. Further, the pregnancy rates in unsynchronised animals were significantly lower in the groups inseminated with sex sorted compared to unsorted semen. The reports from other studies also showed a reduction of pregnancy rates after artificial insemination with sex sorted semen, but mostly in all studies this reduction of fertility as compared to unsorted semen was within 90% (SEIDEL, JR. et al. 1999; SEIDEL et al. 1999). A high reduction of pregnancy rates after artificial insemination with sex sorted semen in this study could be due to reduced quality of the sorted semen or to management failure. Since the preparation and the quality of the sorted semen used for artificial insemination in this study did not marginally differ from these two parameters as reported by other authors (SCHENK et al. 1999; SEIDEL, JR. et al. 1999), it is not likely that this would effect the pregnancy rates to a large extend as obtained in our study. The pregnancy rates in synchronized animals were significantly lower compared to animals inseminated in normal oestrus, but the difference between sorted and unsorted semen was smaller and not significantly different. Lower pregnancy rates in animals with synchronised oestrus could be due to a lack of synchronization in some animals (PURSLEY et al. 1997). We could not find any significant reduction of overall fertility after artificial insemination of synchronized animals with sorted semen. The reduction of fertility was observed only in bull 1 and in bull 2 fertility was comparable to unsorted semen. These results also indicate the difference between bulls in their resistance to flow-cytometrical sorting. A lower effect of flow-cytometrically sorting on pregnancy rates in synchronised as compared to unsynchronized animals, could be explained by management failure. In our study the animals were inseminated under normal farm conditions and the inseminations with sorted semen were performed at the same time in oestrus as for

unsorted semen. Shorter viability of sorted compared to unsorted semen (HOLLINSHEAD et al. 2003; RATH et al. 2003b) is probably responsible for the reduction of the fertilizing capacity of flow-cytometrically sorted spermatozoa in this study.

4 Chapter 2: Reduction of oxidative stress on bovine spermatozoa during flowcytometrical sorting

4.1 Abstract

The goal of the study was to investigate the effect of antioxidant supplementation on the quality of frozen/thawed flowcytometrically sorted bull spermatozoa. Twelve ejaculates from two Holstein Friesian bulls were sorted according to the Beltsville Sperm Sexing Technology. Each ejaculate was divided into three parts and processed as unsorted control, according to a standard sorting protocol and in the presence of different antioxidants (S-AO). Cooling and freezing of the samples was performed equally for all three groups, except that antioxidants were added to TRIS-egg-yolk freezing extender for those semen samples that were already sorted in the presence of antioxidants. The semen quality in frozen/thawed samples was determined by morphology analysis immediately after thawing, motility estimation in a thermo-resistance test after 0, 6, 12 and 24 h incubation at 37°C and FITC-PNA/PI staining after 0, 12 and 24 h of incubation at 37°C. Assessment of motility showed significantly higher ($P<0.05$) percentage of motile spermatozoa in S-AO samples in comparison to unsorted frozen/thawed control at 0, 6 and 24 hours after thawing and compared to normally sorted samples at all times after thawing. Percentage of damaged acrosomes was significantly lower ($P<0.05$) in S-AO samples in comparison to unsorted control (20.8 ± 6.9 % and 30.3 ± 12.0 % respectively). The percentage of morphologically abnormal spermatozoa in this group was significantly lower ($P<0.05$) in comparison to unsorted control and normally sorted samples (25.8 ± 5.2 %; 36.0 ± 12.5 % and 35.1 ± 7.4 %, respectively). Analysis of frozen/thawed spermatozoa with FITC/PI revealed no significant difference in membrane integrity at 0 and 12 hours after sorting, but after 24 hours incubation the S-AO samples had significantly higher ($P<0.001$) percentage of spermatozoa with intact membranes in comparison to unsorted control and normally sorted semen (40.7 ± 6.3 %; 7.8 ± 4.7 % and 7.4 ± 4.6 % respectively). Percentage of acrosome reacted spermatozoa was significantly lower ($P<0.05$) in the S-AO samples in comparison to unsorted controls (14.1 ± 7.5 %, 23.4 ± 5.4% and 28.8 ± 6.3 % vs. 25.9 ± 14.4 %, 38.5 ± 16.7 % and 79.8 ± 4.1 % respectively) for 0, 12, 24 h after thawing and in comparison to normally sorted semen 24 hours after thawing (67.3 ± 10.0 %). This study shows for the first

time the highly beneficial protective effect of antioxidative substances on the quality of flowcytometrically sorted frozen/thawed bull semen.

4.2 Introduction

Membranes of mammalian spermatozoa are rich in highly unsaturated fatty acids and therefore very sensitive to oxygen induced damages (SIKKA 1996). Oxidation of the lipids plays a crucial rule in the function of spermatozoa. Mild peroxidation of the membrane lipids is under normal conditions necessary for capacitation of spermatozoa, which is an important prerequisite for the acrosome reaction and successful fertilisation of the oocyte (KODAMA et al. 1996). Manipulation of the spermatozoa can also cause an increase in lipid peroxidation and leads to irreversible damages of spermatozoa i.e. membrane damages, especially of the acrosome and inhibition of motility (DE LAMIRANDE and GAGNON 1992; WHITTINGTON and FORD 1998). Cooling, long term liquid preservation and freezing of semen are some examples that may cause increased lipid peroxidation (BILODEAU et al. 2000; CALAMERA et al. 2001; CHATTERJEE and GAGNON 2001) .

Although there is no direct evidence that flowcytometrical sperm sorting causes lipid peroxidation, detailed analysis of literature revealed some critical aspect. Labelling of spermatozoa with Hoechst 33342 is necessary to detect small differences in the amount of DNA between X- and Y-chromosome bearing spermatozoa. Usually spermatozoa are incubated with the stain at 34-37°C for 1-1.5 hours, in order to achieve uniform labelling of the DNA. At similar temperatures in mouse and rabbit spermatozoa are known to activate high levels of energy for peroxidation processes (ALVAREZ and STOREY 1985). Spermatozoa are exposed to high pressure and laser light. Negative effects of high pressure on post thaw motility and fertility of flowcytometrically sorted bovine spermatozoa have been reported (SUH and SCHENK 2003) but a direct link to peroxidation was not discussed. UV-Laser light is also known to damage gametes and embryos. Exposure of spermatozoa to 780-nm diode laser inhibited Ca^{2+} uptake by sperm mitochondria and enhanced Ca^{2+} binding to sperm plasma membranes (LUBART et al. 1997). This could cause transient changes in the cytoplasmic Ca^{2+} concentration, which has a regulatory role in control of motility and acrosome reaction (LUBART et al. 1992). Embryos labelled with

Hoechst 33342 died very fast when exposed to permanent UV-light. Fortunately, the exposure of spermatozoa to UV light during the sorting process is only about a 30000^{th} of a second, possibly short enough to avoid sperm damages. Analysis of single spermatozoa, as necessary for flowcytometrical sorting, demands high dilution and consequently a concentration of cells after the sorting process by centrifugation. The natural defence against oxidation provided by seminal plasma is prevented by high dilution during sorting with sheath fluid. Addition of seminal plasma to samples during and after sorting has protective effects on the viability of spermatozoa (MAXWELL et al. 1996).

Centrifugation after sorting also presents stress to spermatozoa and can cause an increase in lipid peroxidation (SHEKARRIZ et al. 1995). This may be more obvious if spermatozoa are stored in liquid nitrogen that increases peroxidation of membrane lipids itself (CHATTERJEE and GAGNON 2001).

Spermatozoa can be protected against lipid peroxidation and different antioxidant substances like Catalase and sodium pyruvate have been used successfully for protection of bull spermatozoa (ASKARI et al. 1994; BECONI et al. 1993; BILODEAU et al. 2002; CALAMERA et al. 2001).

4.3 Material and methods

4.3.1 Methods

Semen was collected from two fertility proven and performance tested Holstein Friesian bulls. Ejaculates were collected with an artificial vagina and kept in a water bath at 27°C. Motility was estimated under phase contrast microscope at 37°C. Concentration of the raw semen was determined with a Thoma® counting Chamber. One part of the raw semen was diluted with Tris-BSA-sample fluid, with (group: +AO) and without antioxidants (1 mM sodium pyruvate and 15 IU/ml Catalase), to $100x10^6$ spermatozoa in 1 mL. Diluted samples were labelled with 15, 20 or 25 µL of an 8.9 mM Hoechst 33342 solution and incubated at 34°C for 1 hour. Labelled and incubated semen samples were kept at 22°C in the dark and sorted within 7 hours after onset of incubation. The concentration of Hoechst 33342 stain giving the best resolution of two sperm populations was used for daily sorting. The other part of the

ejaculates was prepared by standard freezing protocols and used for controls. Briefly, raw semen was diluted with freezing extender I to a concentration of $26.4x10^6$ spermatozoa per millilitre and cooled to 5°C within 2 hours. Semen was then diluted with freezing extender II, supplemented with 0.75 % detergent (Equex STM®, Nova Chemical sales, Inc., Scituate, USA), to a concentration of $13.2x10^6$ spermatozoa/mL. Plastic straws (0.25ml; Minitüb, Tiefenbach, Germany) were filled with 3.3 millions spermatozoa and frozen as described under 3.3.1.

4.3.2 Processing of the sorted spermatozoa

Sperm sorting was performed as described above (3.3.2) using Sodium citrate sheath fluid, optionally supplemented with 100 mg /5 L sodium pyruvate and 15 IU/1 mL catalase (group: +AO). Spermatozoa were sorted into collection tubes, prefilled with 0.5 mL TEST-yolk extender. After collection of 8 millions spermatozoa per tube (approximately 10 mL) cells were centrifuged at 838 x g for 20 minutes. Supernatant was removed and the remaining sperm pellet was diluted with TRIS freezing extender I, optionally supplemented with 1 mM Na-Pyruvat and 15 IU/ mL Catalase (group: +AO) to a concentration of $41x10^6$ spermatozoa/mL and cooled to 5°C within 2 hours. At 5°C were semen samples further diluted with TRIS freezing extender II including 0.75 % detergent (Equex STM®, Nova Chemical sales, Inc., Scituate, USA) and optionally supplemented with 1 mM Na-Pyruvat and 15 IU/ml Catalase (group: +AO) to a concentration of 20.5 $x10^6$ spermatozoa/mL. Plastic straws (0.25mL; Minitüb, Tiefenbach, Germany) were pre-filled with one segment of 50µL of 1+1 mixture made from TRIS freezing extender I and II, in order to close the PVP cotton plug without loosing spermatozoa and were then loaded with a second segment containing 160 µL medium with 3.3 millions sorted spermatozoa. Straws were closed sealed by ultrasound and frozen as described under 3.3.1.

4.3.3 Analysis of frozen thawed samples

4.3.3.1 Motility analysis

Motility was analysed directly after semen collection, before and after sorting as well as after thawing of the frozen samples under a phase-contrast microscope (100 x magnification) equipped with heating plate at 37°C. Additionally motility was tested in

a thermo-resistance test at 37°C over a 24 h period (0 h, 6 h, 12 h and 24 h). Two drops per slide and at least three fields per drop were analysed from each sample.

4.3.3.2 Analysis of Morphology

Spermatozoa were fixed in Hancock fixative and analysed under a phase-contrast microscope (Olympus BX 60, Hamburg, Germany) at 1000x magnification. Morphological abnormalities were separately monitored for acrosomes and for morphologically abnormal spermatozoa (MAS).

4.3.3.3 Acrosome integrity and membrane stability of spermatozoa (FITC-PNA/PI)

Additionally sperm membrane and acrosome integrity was assessed as described previously by WAY and KILLIAN (2002), with some modifications of the protocol. Briefly, pre-warmed Eppendorf cups were filled with 50 µL semen and 1 µL FITC-PNA (2 mg FITC-PNA in 2 mL normoosmolaric PBS) and 2 µL PI solution (1.50 mM 3,8-Diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide and 154 mM NaCl in distillate water) were added. Cups were then incubated at 39°C for 5 minutes. The incubated samples were then supplemented with 10 µL paraformaldehyde (1 % in PBS) and analysed immediately. At least 200 spermatozoa in two drops were examined under a phase contrast and fluorescence microscopy (Olympus BX 60, Hamburg, Germany) at 400 x magnification using a U-MNIB filter. Spermatozoa were divided into four groups: 1. PNA-negative/PI-negative; 2. PNA-negative/PI-positive; 3. PNA-positive/PI-positive; 4. PNA-positive/PI-negative. Mean percentages of viable spermatozoa with intact membranes (group 1) and acrosome reacted spermatozoa (group 3 and 4) are presented in the results.

4.3.3.3 Statistical analyses

Statistical analyses were performed with SIGMA STAT 2.03 for windows (Jandel Scientific Cooperation, San Ragael, CA, USA). The effect of the group on the quality of the semen was analysed for normal distribution and tested with One-way ANOVA or ANOVA on Ranks and Tukey test. Three-way ANOVA was used for analysis of joint effects from bull, group and time after thawing on the quality of the semen. Data

are expressed as percentages or means ± SD. Differences were considered to be significant at $P<0.05$.

4.4 Results

Immediately after thawing and after six hours of incubation at 37°C motility in sorted samples without antioxidative substances (-AO) was significantly lower compared to unsorted controls and to samples sorted and frozen in the presence of antioxidants (+AO). Further incubation at 37°C for 12 hours revealed higher motility in +AO sorted samples compared to sorted samples (-AO). At 24 hours after thawing, the motility was significantly higher in +AO sorted samples compared to -AO samples and unsorted controls. Statistical analysis with three-way ANOVA showed significant effects of the bull on post thaw motility of spermatozoa ($P<0.001$), but in both bulls the motility in +AO sorted samples was significantly higher compared -AO sorted samples ($P<0.05$).

Table 4-1: Percentage of total motile spermatozoa immediately after thawing and after further incubation on 37°C over a 24 h period

n=12	0 h (%)	6 h (%)	12 h (%)	24 h (%)
Control	61.7 ± 4.4^{c}	40.8 ± 14.7^{c}	$19.6 \pm 19.9^{(L)}$	0.0 ± 0.0^{a}
Sorted -AO	53.8 ± 6.1^{d}	$24.6 \pm 12.5^{d,a}$	$5.4 \pm 6.2^{(L)c}$	0.0 ± 0.0^{a}
Sorted +AO	59.6 ± 5.0^{c}	47.1 ± 12.3^{b}	27.5 ± 13.7^{d}	$9.6 \pm 4.5^{(L)b}$

Values with superscripts are significantly different: a:b $P<0.001$; c:d $P<0.05$.

(L) - only local motile spermatozoa were observed.

Frozen/thawed samples sorted in the presence of antioxidants (+AO) had significantly lower percentage of damaged acrosomes in comparison to unsorted control and lower percentage of MAS in comparison to unsorted control and -AO sorted samples.

Table 4-2: Percentage of damaged acrosomes (DA) and morphologically abnormal spermatozoa (MAS)

n=12	DA (%)	MAS (%)
Control	30.3 ± 12.0[a]	36.0 ± 12.5[a]
Sorted -AO	29.0 ± 7.8	35.1 ± 7.4[a]
Sorted +AO	20.8 ± 6.9[b]	25.8 ± 5.2[b]

Values with different superscripts differ significantly (P<0.05).

Immediately after thawing and after 12 hours of incubation at 37°C the difference among viable spermatozoa between all tested groups was not statistical significant. After further incubation up to 24 h, the percentage of viable spermatozoa was significantly higher in the +AO sorted group in comparison to -AO sorted samples and unsorted controls.

Table 4-3: Percentage of viable spermatozoa with intact membranes immediately after thawing and after further incubation at 37°C

n=12	0 h (%)	12 h (%)	24 h (%)
Control	56.0 ± 12.9	44.9 ± 14.9	7.8 ± 4.7[a]
Sorted -AO	53.5 ± 8.6	42.8 ± 8.7	7.4 ± 4.6[a]
Sorted +AO	57.4 ± 6.9	50.0 ± 5.7	40.7 ± 6.3[b]

Values with different superscripts differ significantly (P<0.001).

Percentage of acrosome reacted spermatozoa was significantly lower in AO sorted samples in comparison to unsorted control immediately after thawing and also after further incubation on 37°C for 12 and 24 hours. Significantly lower percentage of acrosome reacted spermatozoa in AO sorted in comparison to normally sorted samples was observed only in the samples after 24 hours incubation on 37°C.

Table 4-4: Percentage of acrosome reacted spermatozoa immediately after thawing and after further incubation at 37°C, according to FITC-PNA/PI staining

n= 12	0h (%)	12h (%)	24h (%)
Control	25.9 ± 14.4[c]	38.5 ± 16.7[c]	79.8 ± 4.1[a]
Sorted -AO	15.5 ± 7.8	29.6 ± 7.1	67.3 ± 10.0[a]
Sorted +AO	14.1 ± 7.5[d]	23.4 ± 5.4[d]	28.8 ± 6.3[b]

Values with different superscripts are significantly different.

a:b $P<0.001$; b:c $P<0.05$

4.5 Discussion

Many factors such as bull effect, semen quality, number of inseminated spermatozoa, reproductive status of the female subjected to insemination, time and site of insemination, inseminator farm management and others affect fertility results. The most important factor is however, the fertilizing ability of the sorted spermatozoa (SEIDEL, JR. et al. 1996; SEIDEL, JR. et al. 1997; VAZQUEZ et al. 2003). It has a direct impact on the economic calculation as shown in a recent survey by SEIDEL (2003) and will be crucial for the decision whether to use the technique in breeding concepts or not. Many attempts have been made to cover low sperm quality by changing the time and the site of insemination. In our opinion, the only way to establish the sorting technology for AI industry is to improve its robustness especially by increasing the lifetime of sorted spermatozoa. Sources to violate spermatozoa during and after sorting have been discussed above. A major source seems to be the production of reactive oxygen species (ROS). Oxygen radicals are known to cause a decrease in motility, pre-capacitation and damages of the membrane system by lipid oxidation, especially when the seminal plasma content is reduced after extensive dilution and washing of spermatozoa (DE LAMIRANDE and GAGNON 1992; ICHIKAWA et al. 1999; OEHNINGER et al. 1995). Several steps in sorting process lead to ROS production, but their negative effect on lipid peroxidation can be minimised by medium supplementation with antioxidative substances (BECONI et al. 1993; MAXWELL and STOJANOV 1996; PENA et al. 2003).

To address this issue, essential functions of sorted frozen/thawed bovine spermatozoa were tested, and compared an extender that are usually applied for flowcytometrical sperm sorting and freezing with a new extender supplemented with antioxidants. The results were also compared to unsorted frozen/thawed controls obtained from the same ejaculate. Sperm motility, morphology, acrosome integrity and membrane stability were assessed in frozen/thawed semen immediately after thawing and after further incubation on 37°C for 24 hours.

In this study, we showed for the first time the successful protection of sorted frozen/thawed spermatozoa by antioxidants. The percentage of motile spermatozoa immediately after thawing and after thermo-resistance test at 37°C was significantly different between groups. In the presence of antioxidants motility of sorted

spermatozoa was always significantly higher than in normally sorted samples and also better than unsorted controls at 0, 6 and 24 h after thawing. This confirms data of unsorted semen, when sperm motility was stabilized in the presence of antioxidants (BILODEAU et al. 2002; DE LAMIRANDE and GAGNON 1992).

In addition, medium supplementation with antioxidants had a positive effect on sperm morphology. Sorted spermatozoa had significantly less damaged acrosomes in comparison to unsorted controls and significantly less morphological abnormalities (MAS) compared to normally sorted semen. In the presence of antioxidants, sorted spermatozoa were even better than unsorted controls, which may also be due to the counterstaining of samples with food dye prior to sorting. This helps to eliminate membrane deficient spermatozoa from sorting (JOHNSON et al. 1999).

Membrane and acrosome integrity of spermatozoa were also assessed with FITC-PNA/PI staining. Membrane integrity of spermatozoa did not differ between the groups up to 12 hours after thawing, but further incubation at 37°C for 24 hours revealed significantly more spermatozoa with normal membrane integrity and intact acrosomes in the presence of antioxidants as compared to normally sorted spermatozoa and unsorted controls. As all conditions before and during sorting were identical except for the presence or absence of antioxidative substances, results indicate that ROS triggered lipid peroxidation is very likely the most harmful part during sorting. Results of this laboratory study have to be verified by test inseminations.

5 Chapter 3: Insemination trial with sex sorted fresh bovine spermatozoa processed in the presence of antioxidative substances

5.1 Abstract

Flowcytometrically sex sorted spermatozoa are still reduced in their fertilising capacity when stored either in cooling extender or after freezing in liquid nitrogen. So far, preservation of sorted spermatozoa differs only marginally from procedures used for unsorted semen. In the present study modifications of a TRIS extender were used to balance major cell damages caused by the sorting process and liquid storing of the sorted spermatozoa. In order to prolong lifetime of sorted spermatozoa a special designed combination of antioxidants (AO) and bovine serum albumin (BSA) were used. This new extender increased the quality of sorted semen and no significant differences were seen in motility between unsorted controls and samples, sorted in the presence of AO and BSA. Similarly, membrane integrity as tested by FITC-PNA/PI did not differ significantly to controls. Acrosome integrity was significantly better in AO/BSA sorted group 24 and 48 hours after incubation at 15°C ($P<0.05$) and also after 48 hours in the AO group without BSA ($P<0.05$) in comparison to unsorted control semen. There was no significant difference in pregnancy rates between unsorted control (66.7 %) and both sorted groups (AO/BSA (58.1 %) and AO (54.5 %)) when semen was inseminated in non-synchronised heifers. Additionally, we could show for the first time that liquid sorted semen can be inseminated successfully within 72 hours after sorting.

5.2 Introduction

Sorting of mammalian spermatozoa has been established at least on laboratory level (for example: JOHNSON et al. 1989; JOHNSON 1991; RATH et al. 1997; SEIDEL, JR. et al. 1996; WELCH and JOHNSON 1999). In the bovine, first companies have already started to offer sexed spermatozoa commercially. So far the sexed spermatozoa are hampered in the quality and lifespan, especially when frozen after the sorting process (HOLLINSHEAD et al. 2002; SEIDEL, JR. et al. 1997). Recently, as shown in chapter 2, we improved the quality of sexed spermatozoa by supplementing TRIS extender with antioxidative substances. However, as the sperm number of sexed semen is highly reduced in comparison to normal AI dosages, inseminations with sorted fresh semen might have more potentials than with sorted frozen semen samples. Insemination doses can then be reduced from 15 to 2 millions sperm with less effect on pregnancy rates (VISHWANATH et al. 1996). Unsorted fresh semen is normally used within three days (VERBERCKMOES et al. 2005). Artificial insemination with sorted semen in different animal species, performed immediately after sorting showed acceptable and with the unsorted control semen comparable pregnancies rates (BUCHANAN et al. 2000; LINDSEY et al. 2002; RATH et al. 2003a). However, with prolonged intervals from sorting to insemination sorted spermatozoa rapidly loose their fertilizing ability. The use of sorted semen is compared to unsorted liquid stored semen limited to a few hours after sorting (SEIDEL, JR. et al. 1999; SEIDEL, JR. et al. 1996). A prolonged fertilizing capacity of liquid stored sex sorted spermatozoa would be for many circumstances superior to frozen/thawed semen.

5.3 Material and Methods

5.3.1 Methods

Semen was collected from a fertility proven and performance tested Holstein Friesian bull. Ejaculates were kept in a water bath at 27°C and motility was estimated under phase contrast microscope (Olympus BX 60) at 37°C. Morphology was analysed after fixation in Hancock fixative, within 4 hours after semen collection. Concentration of spermatozoa in ejaculates was determined with a haemocytometer (Coulter counter®). The raw semen was than diluted with modified Tris-sample fluid (mTSA

and mTSB) to 100x10^6 spermatozoa/ mL. Basic Tris-sample fluid was either supplemented with 15 IU/mL catalase and 1 mM sodium pyruvate (mTSA) or with 15 IU/ml catalase, 1 mM sodium pyruvate, and 3 mg/mL BSA fraction V (mTSB). Diluted samples were labelled with 15, 20 or 25 µL of a 8.12 mM Hoechst 33342 solution and incubated during transport to the laboratory in electrically warmed container (Embryonentransportgefäss® mit digitaler Temperaturkontrolle, Minitüb, Germany). Samples diluted with mTSA were incubated at 37°C for 1.5 hours and those diluted with mTSB were incubated at 34°C for 1 hour. Different temperatures and incubation times were necessary to obtain suitable DNA related labelling of spermatozoa.

5.3.2 Processing of flowcytometrically sorted spermatozoa

Sperm sorting was performed as described in section 3.3.2. Sodium citrate sheath fluid was supplemented with 100 mg /5 L sodium pyruvate and 15 IU/ml catalase. Labelled semen samples were kept at 22°C in the dark and were sorted within 7 hours after onset of incubation. Sorted spermatozoa were centrifuged at 840xg for 20 minutes immediately after collection of 8 millions sorted spermatozoa per collection tube. The supernatant was discharged and the pellet was resuspended with an egg-yolk based fresh semen extender containing 15 IU/ml catalase. Code labelled 0.25 ml plastic straws (Minitüb, Tiefenbach, Germany) were filled with 3x10^6 spermatozoa, sealed and kept at 15°C until analysis or artificial insemination. Straws with sorted spermatozoa and unsorted controls were at the end of sorting day randomly distributed to two experienced AI technicians. All straws were kept in cooling boxes at 15°C until AI. Control samples were prepared similar as sorted samples. Briefly, raw ejaculate was diluted with the same extender as used for dilution of sex sorted spermatozoa. The straws were filled with 3x10^6 spermatozoa, sealed, coded and kept at 15°C until analysis or artificial insemination.

5.3.3 Reanalysis of sorting purity

Reanalysis to identify sort purity was performed immediately after sorting. Aliquots of 500,000 spermatozoa were taken and diluted to 1mL with tris-sample fluid, supplemented with 20 µl of a 0.8 mM solution of Hoechst 33342 and incubated for 20 minutes at 37°C. Thereafter, samples were sonicated and filtered as described

before (WELCH and JOHNSON 1999). Reanalysis was performed at 60 events/sec. The analysis of purity was performed by a curve fitting model.

5.3.4 Motility analysis

Motility was evaluated in raw semen, directly after sorting, and 24 and 48 hours after storage at 15°C. Prior to analyses samples were pre-warmed to 37°C for 15 minutes and analysed under a phase-contrast microscope (Olympus BX 60), equipped with heating plate at 37°C. Two drops and at least three fields per sample were evaluated.

5.3.5 Morphology analysis

Morphology was analysed in raw semen samples, and after incubation of the sorted samples and controls at 15°C for 24 h under a phase-contrast microscope (Olympus BX 60) at 1000 x magnification. At least 200 spermatozoa were examined per sample for morphological abnormalities (MAS) and acrosome integrity.

5.3.6 Acrosome integrity and membrane stability of spermatozoa (FITC-PNA/PI)

Acrosome integrity and membrane stability were also analysed with FITC-PNA/PI as described previously (WAY and KILLIAN 2002). Pre-warmed Eppendorf cups were filled with 50 µL of semen and 1 µL FITC-PNA (2 mg FITC-PNA in 2 mL PBS) as well as 2 µL PI (1 mg propidium iodide in 10 mL physiological NaCl solution) were added. Samples were incubated at 38°C for 5 minutes and supplemented with 5 µL paraformaldehyde (1 % in PBS) immediately before microscopic examination. At least 200 spermatozoa were examined under a fluorescence and phase contrast microscope (Olympus BX 60; U-MNIB filter) at 400x magnification. Spermatozoa were divided into four groups: 1. PNA-negative/PI-negative; 2. PNA-negative/PI-positive; 3. PNA-positive/PI-positive; 4. PNA-positive/PI-negative. Mean percentages of viable spermatozoa with intact membranes (group 1) and acrosome reacted spermatozoa (group 3 and 4) are presented in the results.

5.3.7 Inseminations and pregnancy control

Only heifers (n= 77) from different farms in north Germany were included in the field trial. Inseminations were performed 12-24 hours after onset of oestrus into the

uterine horn, ipsilateral to the dominant follicle. Technicians were advised to insert a normal AI catheter under rectal control as deep as possible into the horn but without extra force or strong manipulation. As all straws were labelled with a code number only, inseminators did not know their identification. Pregnancies were controlled 40-60 days after insemination by transrectal examination. All pregnant animals were allowed to go to term. Sex of calves was recorded.

5.3.8 Statistical analyses

Statistical analyses were performed with SIGMA STAT 2.03 for windows (Jandel Scientific Cooperation, San Ragael, CA, USA). Pregnancy rates and sex of the calves were analysed with Chi-square test. Semen quality parameters were tested for normal distribution and either analysed with One-way ANOVA or ANOVA on Ranks and Tukey test. Data are expressed as percentages or means ± SD. Differences were considered to be significant at $P<0.05$.

5.4 Results

Motility in the raw semen was not significantly different from motility of spermatozoa after sorting and subsequent centrifugation, independent from sorting protocol. Further incubation at 15°C for 24 and 48 hours did also not reveal significant difference between unsorted control and sorted samples. However, significant decrease of sperm motility ($P<0.05$) was observed within control samples between raw semen and those incubated for 24 h, within mTSB samples between 0 h and 24 h and within mTSA samples between 0 h and 48 h incubation at 15°C (Table 5-1).

Table 5-1: Percentage of motile spermatozoa in raw ejaculate, immediately after sorting and after 24h and 48h incubation at 15°C

n=9	0h (%)	24h (%)	48h (%)
Control	72.2 ± 2.6^{a}	66.9 ± 5.3^{b}	63.9 ± 4.2
mTSA	69.8 ± 3.6^{a}	64.4 ± 3.2	56.7 ± 5.0^{b}
mTSB	71.3 ± 2.8^{a}	65.6 ± 4.2^{b}	58.9 ± 4.9

Values with different superscripts differ significantly ($P<0.05$).

Morphology of spermatozoa was analysed in raw semen and in samples of all groups after 24 h of incubation at 15°C. Percentage of spermatozoa with damaged acrosomes were significantly lower in raw semen (6.1 ± 3.1 %) as compared to control samples (12.0 ± 3.1 %) stored for 24h at 15°C ($P<0.001$). In addition, sorted spermatozoa incubated in mTSB had significantly less damaged acrosomes (6.2 ± 2.7 %) than unsorted controls (12.0 ± 3.1 %; $P<0.05$). There was no significant difference between unsorted controls and spermatozoa sorted in mTSA (8.4 ± 4.4 %) and between both sorted groups.

These results were confirmed by FITC/PNA/PI analysis. Prolonged incubation of spermatozoa at 15°C for 24 h and 48 h did not significantly affect the viability of spermatozoa. There was also no significant difference in the percentage of viable spermatozoa between unsorted and both groups of sorted semen.

Table 5-2: Percentage of FITC-PNA/PI negative spermatozoa after 24 h and 48 h of incubation at 15°C

n=9	24 h (%)	48 h (%)
Control	83.4 ± 3.4	81.5 ± 2.5
mTSA	80.5 ± 6.6	81.8 ± 3.7
mTSB	81.5 ± 6.4	83.7 ± 4.0

Significantly ($P<0.05$) lower percentage of exposed acrosomes were observed in samples sorted by the mTSB after 24 hours incubation on 15°C and also in all sorted samples 48 hours after sorting in comparison to unsorted controls. Length of incubation had no significant effect on the acrosome stability of spermatozoa.

Table 5-3: Percentage of spermatozoa with exposed acrosomes (FITC-PNA positive) after 24 and 48 hours incubation on 15°C

n=9	24 h (%)	48 h (%)
Control	6.6 ± 1.8 [a]	8.0 ± 1.7 [a]
mTSA	4.6 ± 1.5	4.2 ± 1.2 [b]
mTSB	3.7 ± 1.7 [b]	3.2 ± 1.5 [b]

Values with different superscripts differ significantly (P<0.001).

Pregnancy results based on rectal palpation 30 to 60 days after insemination did not indicate significant differences between groups. Reanalysis of the sorted semen revealed 92.3 % purity for the X population of sorted spermatozoa. Sex of offspring's from sorted groups was recorded in 25 animals so far and 24 (96 %) were female calves.

Table 5-4: Pregnancy rates after artificial insemination with sorted liquid semen at different times after processing

Inseminated at h after processing	Control (n= 24) (%)	mTSA (n= 22) (%)	mTSB (n= 31) (%)
0-24 h	57.1	44.4	50.0
48 h	60.0	62.5	57.1
72 h	100	60.0	100
total	66.7	54.5	58.1

5.5 Discussion

This is the first report of pregnancies after artificial insemination with sorted liquid semen stored up to 72 hours after sorting at 15°C. Normally, liquid semen maintains its fertilising capacity up to 3-5 days when cooled between 5-15°C. For liquid sorted semen, SEIDEL et al. (1997) reported that its fertilizing capacity is limited to a very short period after processing. In their study none of the heifers inseminated with sorted semen and transported at ambient temperature became pregnant, 22.4 % animals became pregnant after transport at 5°C and inseminated within 9 to 13 hours after sorting and only 2.6 % of the heifers were pregnant when inseminations were performed 17 h to 29 h after sorting.

Such low fertility data are due to several reasons. High dilution of ejaculates diminishes compensatory effects within the sperm population (SAACKE et al. 2000). This is combined with a dilution effect on seminal plasma components that are involved in the prevention of oxidative stress and also mediate successful sperm transport through the female genital tract in some species. Individual bull effects may apply (ANDERSSON et al. 2004) and limit the application of certain biotechniques such as freezing and sorting. Accordingly, more spermatozoa are required per insemination dose to maintain fertility and the number of spermatozoa per AI is usually three to four times higher in frozen sperm samples compared to liquid bull semen dosages (VISHWANATH et al. 1996). Several AI centres offer therefore semen from the genetically best, most required bull as liquid semen.

Similarly, as sorting is known to be affected by some critical steps that may diminish sperm survival, the number should be increased for successful AI similarly to frozen semen. Unfortunately, the opposite is true. Even with high speed flowcytometry, the production of sorted spermatozoa with high purity above 90% is limited to 15 million per hour (RATH et al. 2003b) and usually in the bovine not more than $2x10^6$ live spermatozoa are used per insemination, independently whether spermatozoa are used as liquid or frozen/thawed sample (DOYLE et al. 1999; SEIDEL, JR. et al. 1999; SEIDEL et al. 1999; SEIDEL, JR. et al. 1997). As freezing is an additional source of cell stress and cell violation (BILODEAU et al. 2000; CHATTERJEE and GAGNON

2001), it makes sense to use liquid sorted semen for AI, especially, where AI centers have established the infrastructures for liquid semen distribution.

The present results indicate that proper handling and protection before and after sorting of semen from selected bulls leads to pregnancy rates comparable to those from unsorted controls. Further sources reducing viability of sorted spermatozoa are known. For example capacitation was induced by sorting (KNÖPPEL 2001). Capacitation of spermatozoa is triggered by oxidation of lipids in plasma membranes and may even lead to acrosome reaction (RIVLIN et al. 2004). Under normal fertilizing conditions mild peroxidation of plasma membrane lipids are necessary for capacitation of spermatozoa and fertilization of oocytes (OEHNINGER et al. 1995), but when it happens during sperm sorting, it can lead to precapacitation, acrosome loss and decreased motility of spermatozoa (BAUMBER et al. 2000). Data from BILODEAU et al. (2002) indicated positive effects of antioxidants on viability of spermatozoa. Accordingly, the present results verify the protecting function of antioxidative substances when added to the extenders used for sorted spermatoza.

In addition, BSA was beneficial for maintenance of membrane integrity as it increases membrane fluidity of bull spermatozoa (WOLFE et al. 2001). In consequence, BSA protected spermatozoa during incubation with the stain. Interestingly, the uptake of Hoechst 33342 was more homogenous in the presence of BSA. Therefore, temperature and time of incubation could be lowered from 37°C / 1,5 h to 34°C / 1 h. As reported by SIRIVAIDYAPONG et al. (2000) lower temperatures of sperm incubation are less detrimental to acrosome stability. Accordingly, the present study showed fewer incidences of acrosome changes during processing and sorting.

6 Chapter 4: Improvement of sperm sorting efficiency and fertilizing capacity employing two variations of a new bull semen extender (Sexcess®)

6.1 Abstract

The aim of the present study was to analyse the effect of antioxidants and temporary chemical inhibition of sperm motility before and during flowcytometrical sorting on the quality and fertilizing capacity of sex sorted and frozen/thawed spermatozoa. The quality and fertilizing capacity of temporarily immobilized spermatozoa sorted in the presence of sodium fluoride (S-AO-NF) was compared with sorted semen using a standard protocol as developed in our laboratory (S-AO) and with unsorted control (C). Semen from two bulls was used and in total 283 first inseminations with 2 million live, frozen/thawed sex sorted and unsorted spermatozoa were performed on 197 farms. Motility of spermatozoa was completely inhibited after addition of sodium fluoride and after sorting and centrifugation returned to a similar degree as before. Motility after thawing and incubation at 37°C did not differ between groups. After further incubation at 37°C for 12 h and 24 h motility was significantly higher ($P<0.001$) in both sorted groups compared to unsorted semen (37.1 ± 4.9 % vs. 33.1 ± 6.5 % vs. 7.5 ± 11.6 % and 22.9 ± 7.6 % vs. 21.3 ± 10.3 % vs. 0.0 ± 0.0 % for S-AO-NF vs. S-AO vs. C for 12 and 24 hours respectively). The percentage of spermatozoa with acrosome abnormalities in frozen/thawed samples was significantly higher ($P<0.05$) in S-AO and C group when compared to S-AO-NF (23.0 ± 5.2 %, 22.3 ± 4.0 % and 14.7 ± 7.5 % respectively). The same difference was found for morphological sperm abnormalities (30.0 ± 6.7 %, 29.0 ± 5.8 % and 19.0 ± 8.1 % respectively). The percentage of acrosome reacted and viable spermatozoa was analysed by FITC-PNA/PI staining. The percentage of acrosome reacted spermatozoa was significantly lower ($P<0.001$) in both groups of sorted semen when compared to unsorted control (10.6 ± 3.5 % vs. 6.7 ± 2.6 % vs. 19.1±4.7 % for S-AO, S-AO-NF and C respectively). The percentage of viable spermatozoa was significantly higher ($P<0.05$) in the S-AO-NF group when compared to groups S-AO and C (72.1 ± 5.0 % vs. 56.9 ± 4.7 % vs. 61.4 ± 8.2 % respectively). Pregnancy rates after artificial insemination with the frozen/thawed semen were not significantly different between sorted spermatozoa and controls (72.7 % vs. 73.3 % vs. 79.2 % for S-AO-NF, S-AO and C respectively).

6.2 Introduction

Like in other domestic animal species, a broader utilisation of sexed semen would be very beneficial for cattle industry. So far, only flowcytometry allows producing a significant shift of sex ratios. However, the technique still has its limitations in sorted sperm output (RATH et al. 2003a; SEIDEL, JR. et al. 1999), although many improvements have helped in the recent years to start first commercial applications as shown in UK and Texas, USA.

Especially introduction of high speed flowcytometry (JOHNSON et al. 1999), better orientation of spermatozoa in front of the Laser beam (RENS et al. 1998) and improvements in sperm preparation before and after sorting by special media components as shown in the three chapters above (see chapter 3 - 5) allow to minimize the sperm concentration and use liquid as well as frozen/thawed spermatozoa. However, fertilizing potential of sorted frozen/thawed spermatozoa has not reached the quality of unsorted analogues. The lifetime after sorting and freezing/thawing is limited and even with adapted insemination protocols, the number of calves born per AI or number of AI necessary to get the same fertility rates are critical (SEIDEL, JR. and GARNER 2002). A potential solution might be to safe as much energy as possible during processing and sorting. This might be achieved with reversible, chemical inhibition of the sperm metabolism.

Ornidazone (BONE et al. 2000), bromoydroxypropanone (PORTER and JONES 1995), nitric oxide (ROSSELLI et al. 1995), alfa-chlorhydrin (BROWN-WOODMAN and WHITE 1976; COONEY and JONES 1988; STEVENSON and JONES 1985), gossypol (POSO et al. 1980; STEVENSON and JONES 1985), Cytochalasin B (PETERSON and FREUND 1977), formaldehyde (DOTT et al. 1976) and sodium fluoride (FOOTE 2001; WANG et al. 1995) are examples for chemicals, known to have an inhibiting effect on sperm motility. DOTT et al. (1976) were able to achieve pregnancies after artificial insemination in ewes and sows with formaldehyde-immobilized spermatozoa. As the toxicity of formaldehyde is high (CONAWAY et al. 1996), specific inhibition of sperm motility may be performed by sodium fluoride (FOOTE 2001). Sodium fluoride is a conventional inhibitor of protein-thyrosine-phosphatase (ERRASFA and STERN 1994), which is included in the process of sperm capacitation and motility (BALDI et al. 1996; LUCONI et al. 2005) and was

reported as immobilizing substance for bull spermatozoa (SCHOFF and LARDY 1987).

Flowcytometrical sorting could benefit from temporarily inhibition of sperm activity not only for the prolongation of sperm lifetime, but also to get a better orientation of spermatozoa in front of the Laser beam and to reduce individual bull effects during staining and sorting. In the present study, we analyzed the effect of sperm immobilization with sodium fluoride on sorting efficiency, quality and fertility of frozen thawed bull spermatozoa.

6.3 Materials and Methods

6.3.1 Methods

Semen was collected from fertility proven and performance tested Holstein Friesian bull (Bull 1) and a young Limousine bull (Bull 2) in performance test. Ejaculates were kept in a water bath at 27°C and motility was estimated under phase contrast microscope (Olympus BX 60) at 37°C. Morphology was analysed after fixation in Hancock fixative, within 4 hours after semen collection. Concentrations of spermatozoa in ejaculates were determined with a haemocytometer (Coulter counter®). Raw semen was divided into two parts and diluted to $1x10^8$ spermatozoa/mL either with the commercially available extender Sexcess® AX (Nordrind Verden, Germany), a TRIS based extender supplemented with antioxidants and BSA fraction V (group S-AO), or Sexcess® FX containing additionally sodium fluoride (group S-AO-FX). Spermatozoa were labelled with 15, 20 and 25 µL of a 8.12 mM Hoechst 33342 solution and incubated for 1 hour at 34°C. Thereafter, labelled semen samples were kept at 22°C in the dark and sorted within 7 hours after onset of incubation. The labelled semen samples were pre-tested for maximal separation with a flowcytometer and the concentration of Hoechst 33342 stain giving the best resolution of two sperm populations was used for daily sorting. The other part of the ejaculates was frozen following a routine protocol of a commercial AI center and served as control. Briefly, semen was gradually diluted with TRIS egg-yolk freezing extender I to the concentration of $26.4x10^6$ spermatozoa/ml, cooled to 5°C within two hours and further diluted with TRIS egg-yolk freezing extender II including 0.75 % detergent (Equex STM®, Nova Chemical sales, Inc., Scituate, USA)

to a concentration of $13.2x10^6$ spermatozoa / mL or $3.3x10^6$ spermatozoa/straw, filled into 0.25 mL straws (Minitüb, Tiefenbach, Germany), and frozen in liquid nitrogen as described under section 3.3.1.

6.3.2 Processing of the sorted spermatozoa

Semen was sorted as described above (see 5.3.2). Sorted spermatozoa of group S-AO were collected into collection fluid (max. 8 million spermatozoa per tube) and then centrifuged as described before at 838xg for 20 minutes (see 5.3.2). Centrifugation of sorted spermatozoa in group S-AO-NF allowed lower force (500xg for 15 minutes) because cells were immobilized and gave a more distinct pellet. Supernatant was discharged and the remaining sperm pellet of both groups was diluted with extender Sexcess I® (Nordrind Verden, Germany) to a concentration of $41x10^6$ spermatozoa /mL and cooled to 5°C within 2 hours. Once 5°C was reached, semen samples were further diluted with extender Sexcess II® (Nordrind Verden, Germany) to a concentration of $20.5x10^6$ spermatozoa/mL. Plastic straws (0.25 mL) (Minitüb, Tiefenbach, Germany) were pre-filled with a first segment (50 µL of a mixture made from extender I and II), and with a second segment, filled with 160 µL sorted semen (3.3 millions spermatozoa in total, equivalent to 2 millions life spermatozoa). Semen samples were frozen as already described under section 3.3.1. Briefly, straws were placed on metal holder in nitrogen vapour 3-5 cm above LN_2 for 15 minutes. Frozen samples were than plunged into LN_2 and kept in the semen container (in LN_2) until used for analysis or artificial insemination.

6.3.3 Sperm analysis of frozen thawed samples

6.3.3.1 Reanalysis of sorting purity

Reanalysis to identify sort purity was performed after thawing. Aliquots of 1 million spermatozoa were taken and diluted to 1 mL with tris-sample solution, supplemented with 20 µl of a 0.8 mM solution of Hoechst 33342 and incubated for 20 minutes at 37°C. Thereafter, samples were sonicated and filtered as described before (JOHNSON et al. 1999). Reanalysis was performed at 60 events / sec. The analysis of purity was performed by a curve fitting model.

6.3.3.2 Motility analysis

Sperm motility of raw semen, of sperm samples before and after sorting and after thawing was analysed. Prior to analyses samples were pre-warmed to 37°C for 15 minutes and analysed under a phase-contrast microscope (OlympusBX60), equipped with heating plate at 37°C. Two drops and at least three fields per sample were evaluated.

6.3.3.3 Morphology analysis

Morphology was analysed after fixation of the samples in Hancock solution under a phase-contrast microscope (Olympus BX 60) at 1000x magnification. At least 200 spermatozoa were examined per sample for morphological abnormalities (MAS) and acrosome integrity.

6.3.3.4 Acrosome integrity and membrane stability of spermatozoa (FITC-PNA/PI)

Acrosome integrity and membrane stability were analysed with FITC-PNA/PI as described previously (WAY and KILLIAN 2002). Pre-warmed Eppendorf cups were filled with 50 µL of semen and 1 µL FITC-PNA (2 mg FITC-PNA in 2 mL PBS) as well as 2 µL PI (1 mg propidium iodide in 10 mL physiological NaCl solution) were added. Samples were incubated at 38°C for 5 minutes and supplemented with 5 µL paraformaldehyde (1 % in PBS) immediately before microscopic examination. At least 200 spermatozoa were examined under a fluorescence and phase contrast microscope (Olympus BX 60; U-MNIB filter) at 400x magnification. Spermatozoa were divided into four groups: 1. PNA-negative/PI-negative; 2. PNA-negative/PI-positive; 3. PNA-positive/PI-positive; 4. PNA-positive/PI-negative. Mean percentages of viable spermatozoa with intact membranes (group 1) and acrosome reacted spermatozoa (group 3 and 4) are presented in the results.

6.3.4 Artificial insemination and pregnancy control

All the straws were coded in order to make it impossible for inseminators to know the content. Straws were randomly distributed to three well experienced Slovenian AI technicians within 1.5 month after sorting. For AI, semen samples were thawed at

37℃ for 20 sec. and inseminated 12-24 hours after onset of natural oestrus. Technicians were advised to insert a normal AI catheter under rectal control as deep as possible into the uterine horn but without extra force or strong manipulation. In cases, where it was difficult to determine the location and size of the follicle, semen was either deposited into the uterine body or the content of the straw was split for AI in both uterine horns. Pregnancies were controlled 30-60 days after insemination by transrectal examination and transrectal ultrasonography (Aloka®; 5 MHz). All pregnant animals were allowed to go to term.

6.3.5 Statistical analyses

Statistical analyses were performed with SIGMA STAT 2.03 for windows (Jandel Scientific Cooperation, San Ragael, CA, USA). Pregnancy rates and sex of the calves were analysed with Chi-square test. Semen quality parameters were tested for normal distribution and either analysed with One-way ANOVA or ANOVA and Ranks and Tukey test. Data are expressed as percentages or means ± SD. Differences were considered to be significant at $P<0.05$.

6.4 Results

In the raw semen samples were 72.1 ± 3.7 % motile spermatozoa. After labelling and before sorting in the S-AO group were 66.9 ± 4.6 % motile spermatozoa, whereas all spermatozoa in the S-AO-NF group were immobilized. After sorting the percentage of motile spermatozoa in S-AO-NF group was 72.9 ± 4.9 %, whereas sperm motility in S-AO samples was 70.6 ± 5.6%. The difference was not significant ($P<0.43$). Sperm motility after thawing and incubation at 37℃ for 6 h was not significantly different between groups. Further incubation of frozen thawed spermatozoa for 12 and 24 hours showed significant higher motility of spermatozoa in both sorted groups compared to unsorted standard processed semen (Table 6-1).

Table 6-1: Sperm motility of sorted and control groups immediately after thawing (0 h) and after further incubation at 37°C for 6, 12 and 24 h

	0 h (%)	6 h (%)	12 h (%)	24 h (%)
Control (n= 13)	61.5 ± 3.8	45.8 ± 9.3	7.5 ± 11.6$^{a(L)}$	0.0 ± 0.0^{a}
S-AO (n= 8)	59.4 ± 4.2	45.6 ± 9.4	33.1 ± 6.5^{b}	21.3 ± 10.3$^{b(L)}$
S-AO-NF (n= 7)	59.0 ± 5.9	47.9 ± 7.0	37.1 ± 4.9^{b}	22.9 ± 7.6$^{b(L)}$

Values with different superscripts differ significantly (P<0.001)

(L) - only local motile spermatozoa were observed.

After thawing the percentage of spermatozoa with damaged acrosomes and morphologically abnormal spermatozoa were significant lower in S-AO-NF group compared to S-AO group and unsorted control samples (Table 6-2).

Table 6-2: Percentage of spermatozoa with damaged acrosomes (DA) and morphologically abnormal spermatozoa (MAS)

	DA (%)	MAS (%)
Control (n= 13)	22.3 ± 4.0^{a}	29.0 ± 5.8^{a}
S-AO (n= 8)	23.0 ± 5.2^{a}	30.0 ± 6.7^{a}
S-AO-NF (n= 7)	14.7 ± 7.5^{b}	19.0 ± 8.1^{b}

Values with different superscripts differ significantly (P<0.05)

The percentage of acrosome reacted spermatozoa as seen after FITC-PNA staining was significantly lower in both sorted groups as compared to unsorted control samples. The percentage of viable spermatozoa was significantly higher in S-AO-NF group compared to S-AO group and unsorted control (Table 6-3).

Table 6-3: Percentage of acrosome reacted (AR) and spermatozoa with intact membranes (Viable) in frozen/thawed samples, according to FITC-PNA/PI staining

	AR (%)	Viable (%)
Control (n= 13)	19.1 ± 4.7[a]	61.4 ± 8.2[c]
S-AO (n= 8)	10.6 ± 3.5[b]	56.9 ± 4.7[c]
S-AO-NF (n= 7)	6.7 ± 2.6[b]	72.1 ± 5.0[d]

Values with different superscripts are significantly different: a:b $P<0.001$; c:d $P<0.05$

In total 283 inseminations were performed under field conditions on 197 Slovenian farms. Table 4 shows the pregnancy results. No differences were found between sorted and unsorted semen samples or among sorted samples. Reanalysis of semen after thawing revealed 92.5 % average purity for X-chromosome bearing spermatozoa. So far, calves are not born making it impossible to verify the prediction.

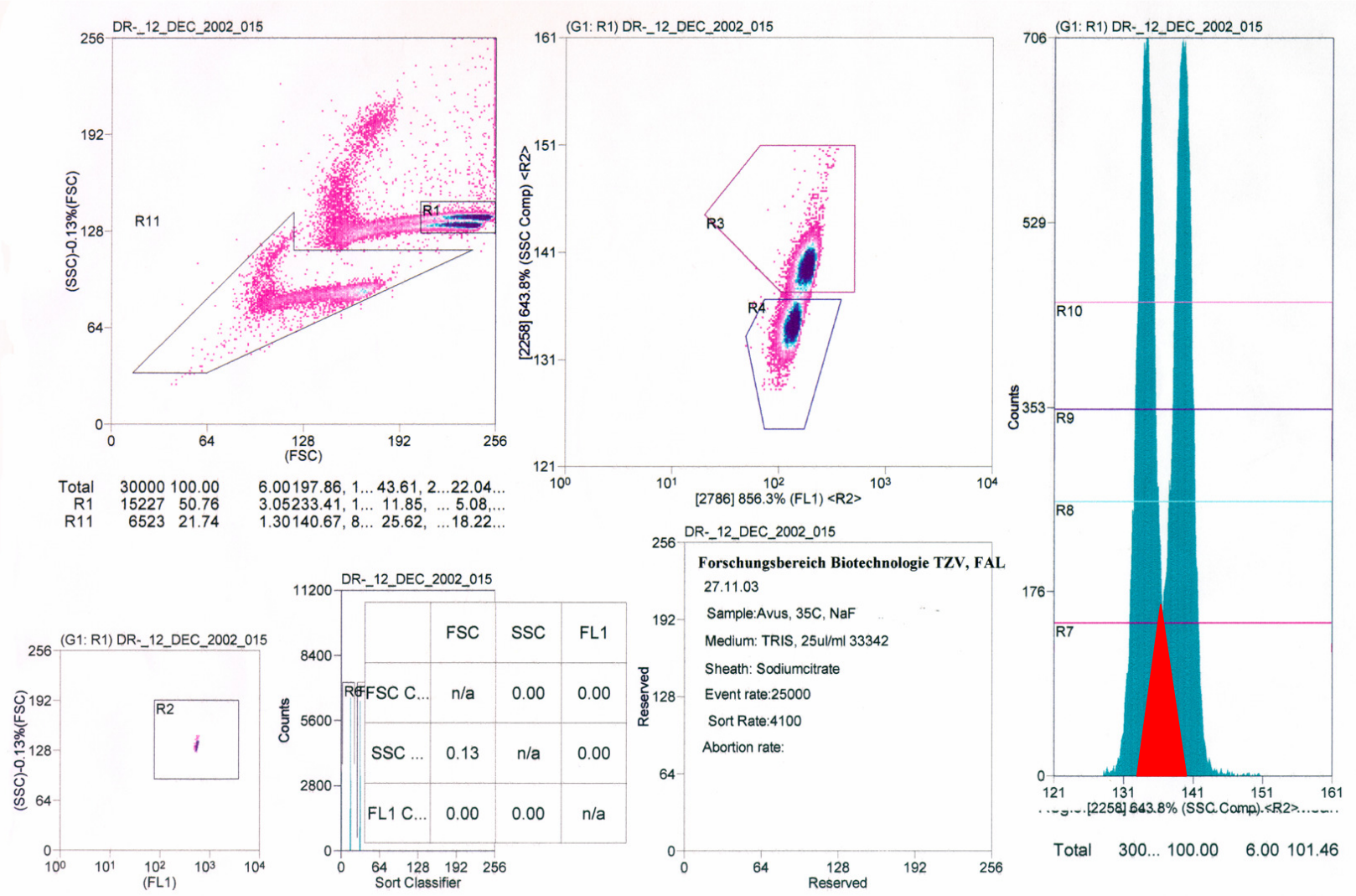
Table 6-4: Pregnancy rates after artificial insemination with sorted frozen/thawed spermatozoa diluted in Sexcess® AX or Sexcess® FX

	Control	Sexcess® AX (S-AO)	Sexcess® FX (S-AO-NF)
Bull 1(%)	75.5 %	73.5 %	62.1 %
Pregnant/all (n/n)	37/49	36/49	36/58
Bull 2 (%)	79.2 %	73.3 %	72.7 %
Pregnant/all (n/n)	42/53	32/44	22/30
Total (%)	77.5 %	73.1 %	65.9 %
Total (n/n)	79/102	68/93	58/88

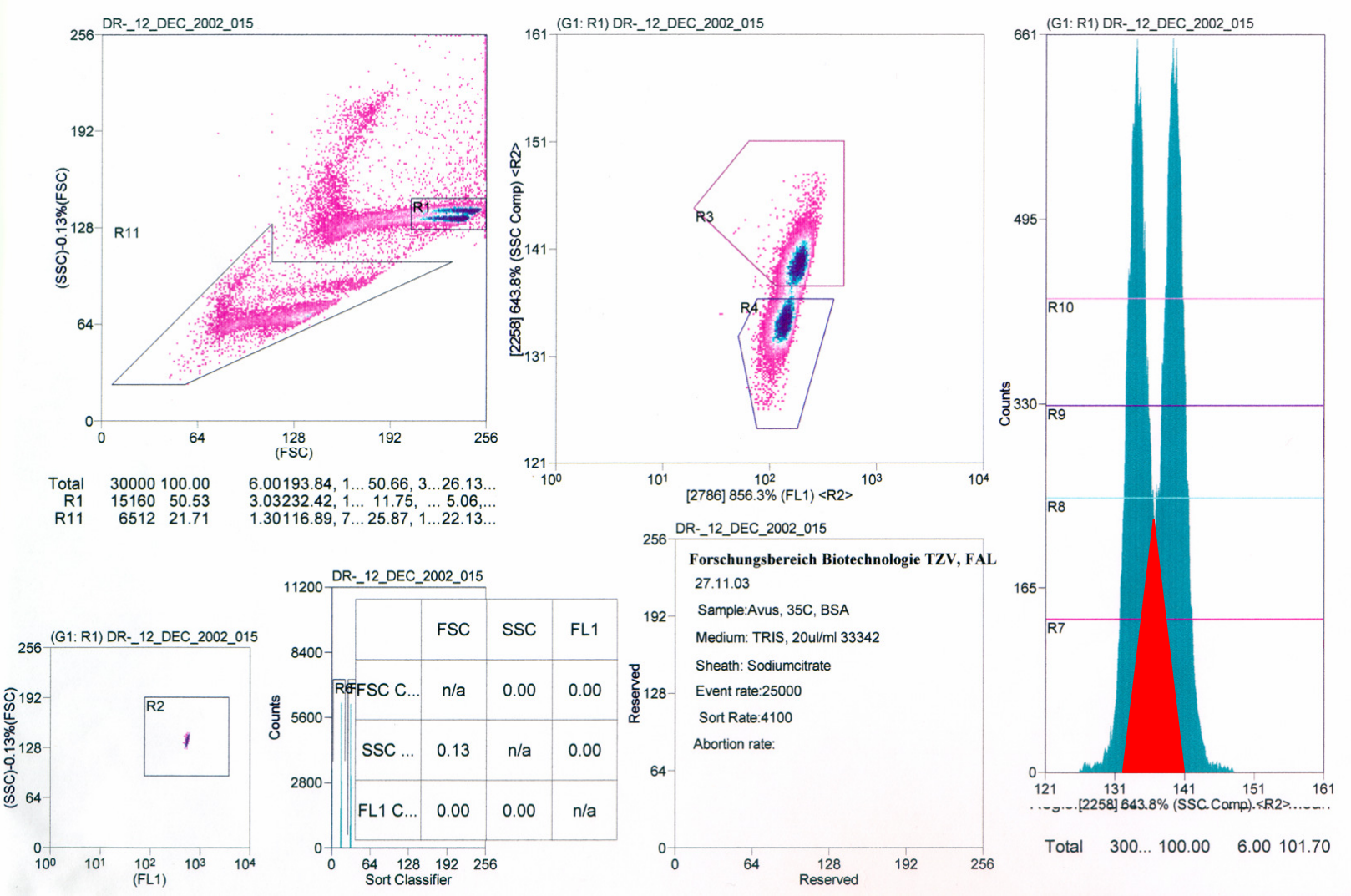
No differences in pregnancy rates were seen among AI technicians (69.1, 70.1 and 77.9 %). Place of semen deposition had a significant effect on the pregnancy rates and were lowest after sperm deposition into the uterine body (66.0 %), compared to insemination into the ipsilateral uterine horn (67.7 %) and compared to those

inseminations, where the content of the straw was divided and inseminated into both horns (82.5 %; $P<0.05$). For all groups no bull specific effect was seen.

The use of Sexcess® FX that immobilizes spermatozoa, promoted the sorting efficiency. During flow-cytometrically sorting, the size of overlapping populations was smaller in the samples containing immobilized spermatozoa (picture 6.1) compared to the samples with motile spermatozoa (picture 6.2).



Picture 6-1: Computer presentation of sperm separation after inhibition of sperm motility with sodium fluoride
Red triangle represents the overlapping populations of X and Y spermatozoa



Picture 6-2: Computer presentation of sperm separation in standard prepared sample
Red triangle represents the overlapping populations of X and Y spermatozoa

6.5 Discussion

In the present study, we have demonstrated that proper handling and protection of the semen during flowcytometrical sorting and freezing leads to high quality of sexed semen and to pregnancy rates that are comparable with those of unsorted controls after artificial insemination under normal field conditions. However, it has to be mentioned that all experiments were performed with semen from selected bulls. Selection criteria were that non return rates were above average and that staining and sorting was homogenous between collection days in order to speed up the sorting process.

The use of Sexcess® FX that immobilizes spermatozoa, promoted the sorting efficiency as the laminar flow was more stable and homogenous compared to motile spermatozoa.

Previous studies demonstrated lower pregnancies rates after artificial inseminations with flowcytometrically sorted spermatozoa when compared to unsorted control. Probable reasons were induction of capacitation, membrane damages (KNÖPPEL 2001; MAXWELL et al. 1998; RATH et al. 1997) and rapid loss of motility (HOLLINSHEAD et al. 2003; RATH et al. 2003b). In consequence, insemination protocols had to be changed and insemination had to be performed closer to ovulation.

In the present study, lifetime of sorted spermatozoa was at least as long as for those in the control group. The percentage of motile spermatozoa was even higher in both groups of sorted semen compared to unsorted controls after incubation at 37°C for 12 and 24 h. This was caused by utilization of antioxidants, preventing generation of reactive oxygen species, oxidation of membrane lipids, and loss of motility due to oxidation (BAUMBER et al. 2000). Addition of sodium fluoride caused a complete, reversible inhibition of sperm motility and seemed to reduce interference of mitochondria in the mid piece of the sperm tail with the high voltage electric field, necessary to separate both sperm populations. Sorted spermatozoa regained their motility after sorting and centrifugation completely, which showes the complete reversibility of motility inhibition with sodium fluoride.

CHINOY and NARAYA (1994) reported toxic effects of sodium fluoride on human spermatozoa, causing irreversible damages like elongated heads, deflagellation, acrosome loss and coiling of the tails. However, in the present study, concentration of sodium fluoride was much lower and enabled spermatozoa to completely regain motility. So far, no negative effects of sodium fluoride were seen. In opposite, the highest sperm membrane integrity was found in those samples that were diluted with Sexcess® FX. In addition, these samples also showed better sperm quality after sorting, which might mainly be due to shorter centrifugation time and force.

Laboratory results were confirmed by pregnancy data that were obtained from an AI field trial in heifers. No significant differences were seen between inseminations with sorted vs. unsorted semen.

The handling was as easy as with unsorted semen and no significant effect on the insemination results were found among AI technician. However, the site of semen deposition in the female genital tract had significant effect on pregnancy rates. WILLIAMS et al. (1988) reported reduced fertility after cervical deposition of the semen compared to uterine body or bicornual insemination, and SENGER et al. (1988) saw significantly more pregnant animals after bicornual insemination compared to insemination into the uterine body. Accordingly, in the present study significantly more pregnant animals became pregnant after bicornual compared to uterine body insemination.

In conclusion, the use of Sexcess® extender to optimize bull sperm quality has successfully been approved and improved the fertilizing ability of sexed spermatozoa to a comparable status to unsorted frozen/thawed bull semen. Further studies will have to analyse more intensively bull effects to broaden the number of males to be included in a sperm sorting based breeding programme.

7 Conclusions

The results from previous studies showed quality and fertilizing deficiencies of standard processed flowcytometrically sorted spermatozoa when compared to unsorted semen. Additionally, as shown in our study, a significant bull effect is not negligible (Chapter 1).

The lifespan of flowcytometrically sorted spermatozoa was shortened significantly when using the so far recommended sperm preparations before, during, and after sorting. In consequence AI had to be performed as close as possible to ovulation and might also include modified AI strategies. However, under field conditions pregnancy rates vary to much as shown in the first part of the present experiments (Chapter 1). Therefore, the major goal of this study was to prolong viability and to increase fertilizing ability of sex sorted spermatozoa.

A major reason for reduced fertilizing potentials of sorted spermatozoa seems to be the formation of oxygen radicals. As consequently shown in chapter 2, a significant improvement of viability and fertilizing capacity was achieved by medium supplementation with antioxidants. Acceptable pregnancy rates were obtained under normal field conditions after storage of liquid semen up to three days (Chapter 3). Modifications of extenders with antioxidants also had a positive effect on the viability of frozen/thawed sex sorted spermatozoa (Chapter 2). Further improvements were made when semen was treated with a new extender (Sexcess®) that includes antioxidants and reversibly inhibits motility during processing. Fixed spermatozoa were more efficiently sortable, viability after thawing was prolonged and this increases the chances for fertilization (Chapter 4).

In conclusion, the new protocol to process and sort bull semen as well as a new concept to store semen samples, increased pregnancy rates to a level that is comparable to unsorted semen. However, these results were obtained from bulls selected for sorting. Before broader use more experiments have to elucidate bull specific effects.

8 Summary

Primož Klinc (2005):

Improved Fertility of Flowcytometrically Sex Selected Bull Spermatozoa

The influence of flowcytometrical sorting and the effect of different modifications of extenders on the viability and fertilizing capacity of liquid and frozen/thawed bull spermatozoa were analysed in the present study. The study was divided in four experimental parts.

The aims of the first study were to analyze effects of semen processing after sorting and subsequent freezing in liquid nitrogen on the quality and fertilizing capacity of bull spermatozoa. Semen was obtained from two fertility proven Holstein Friesian bulls. Quality of the frozen/thawed semen was evaluated by motility estimation, morphology analysis, membrane stability (6-CFDA/PI) test, capacitation test (FITC-PNA/PI staining with L-α-Lysophosphatidylcholine), and fertility assessment in an insemination trial on farm. A prolonged period after high dilution of sorted spermatozoa before freezing had a significantly negative effect on motility ($P<0.001$) and capacitation status ($P<0.05$). Positive effects on membrane stability were seen, when glycerol was added at 5°C shortly before straws were frozen, compared to a system where glycerol was added before the equilibration process at room temperature ($P<0.001$). Independently from sperm processing after sorting, sexed spermatozoa had significantly more damaged acrosomes and morphological abnormalities ($P<0.001$). In addition, sex sorted frozen/thawed spermatozoa (immediate centrifugation and glycerol addition at 5°C) and unsorted frozen/thawed semen samples were submitted to a thermo-resistance test at 37°C. Immediately after thawing no significant difference was seen in the percentage of motile spermatozoa between sorted and unsorted semen samples. However, after further incubation at 37°C, motility dropped significantly after 3 h ($P<0.001$) and 6 h ($P<0.05$). The fertilizing capacity of sex-sorted and unsorted spermatozoa from same ejaculates was tested in a field trial in heifers and cows with natural or synchronised oestrus cycle, respectively. In natural oestrus, more animals became pregnant after artificial insemination with unsorted than with sex-sorted spermatozoa (56.5 % vs. 17.6 %; $P<0.001$). No significant differences were observed between unsorted and

sex-sorted frozen/thawed semen samples after artificial insemination of the animals with synchronized estrus (36.4 % vs. 21.3 %).

The goal of the second study was to investigate the effect of antioxidant supplementation on the quality of frozen/thawed sexed bull spermatozoa. Twelve ejaculates from two Holstein Friesian bulls were sorted according to the Beltsville Sperm Sexing Technology. Each ejaculate was divided into three parts and processed as unsorted control, according to a standard sorting protocol and in the presence of different antioxidants (S-AO). Cooling and freezing of the samples was performed equally for all three groups, except that antioxidants were added to TRIS-egg-yolk freezing extender for those semen samples that were already sorted in the presence of antioxidants. The semen quality in frozen/thawed samples was determined by morphology analysis immediately after thawing, motility estimation in a thermo-resistance test after 0, 6, 12 and 24 h incubation at 37°C and FITC-PNA/PI staining after 0, 12 and 24 h of incubation at 37°C. Assessment of motility showed significantly more ($P<0.05$) motile spermatozoa in S-AO samples as compared to unsorted frozen/thawed controls at 0, 6 and 24 h after thawing and compared to normally sorted samples at all times after thawing. Percentage of damaged acrosomes was significantly lower ($P<0.05$) in S-AO samples in comparison to unsorted control (20.8 ± 6.9 % and 30.3 ± 12.0 %, respectively) and the percentage of morphological abnormal spermatozoa was in this group significantly lower ($P<0.05$) in comparison to unsorted control and normally sorted samples (25.8 ± 5.2 %; 36.0 ± 12.5 % and 35.1 ± 7.4 %, respectively). Analysis of frozen/thawed spermatozoa with FITC/PI revealed no significant difference in membrane integrity at 0 and 12 hours after sorting, but after 24 hours incubation the S-AO samples had significantly higher ($P<0.001$) percentage of spermatozoa with intact membranes in comparison to unsorted control and normally sorted semen (40.7 ± 6.3 %; 7.8 ± 4.7 % and 7.4 ± 4.6 % respectively). Percentage of acrosome reacted spermatozoa was significantly lower ($P<0.05$) in the S-AO samples in comparison to unsorted controls (14.1 ± 7.5 % vs. 25.9 ± 14.4 %; 23.4 ± 5.4 % vs. 38.5 ± 16.7 % and 28.8 ± 6.3 % vs. 79.8 ± 4.1 % respectively) for 0, 12, 24 h after thawing and in comparison to normally sorted semen 24 hours after thawing (67.3 ± 10.0 %). This study shows for the first time the highly beneficial protective effect of antioxidative substances on the quality of sexed, frozen thawed bull semen.

In the third study, modifications of a TRIS extender were used to balance major cells damages caused by the sorting process and liquid storing of the sorted spermatozoa. In order to prolong lifetime of sorted spermatozoa a special designed combination of antioxidants (AO) and bovine serum albumin (BSA) was used. The new extender increased the quality of sexed semen and no significant difference were seen in motility between unsorted controls and samples, sorted in the presence of AO and BSA. Similarly, membrane integrity as tested by FITC-PNA/PI did not differ significantly to controls. Acrosome integrity was significantly better in AO/BSA sorted group 24 and 48 hours after incubation at 15°C ($P<0.05$) and also after 48 hours in the AO group without BSA ($P<0.05$) in comparison to unsorted control semen. There was no significant difference in pregnancy rates between unsorted control (66.7 %) and both sorted groups (AO/BSA (58.1 %) and AO (54.5 %) when semen was inseminated in non-synchronised heifers. Additionally, we could show for the first time that liquid sorted semen can be inseminated successfully within 72 hours after sorting.

The aim of the fourth study was to analyse the effect of antioxidants and temporary chemical inhibition of sperm motility before and during flowcytometrical sorting on the quality and fertilizing capacity of sex sorted and frozen/thawed spermatozoa. The quality and fertilizing capacity of temporarily immobilized spermatozoa sorted in the presence of sodium fluoride (S-AO-NF) was compared with sorted semen using a standard protocol as developed in our laboratory (S-AO) and with unsorted control (C). Semen from two bulls was used and in total 283 first inseminations with 2 million live, frozen/thawed sex sorted and unsorted spermatozoa were performed on 197 farms. After addition of sodium fluoride the motility of spermatozoa was completely inhibited and after sorting and centrifugation returned to a similar degree as before. Motility after thawing and incubation at 37°C did not differ between groups. After further incubation at 37°C for 12 h and 24 h motility was significant higher ($P<0.001$) in both sorted groups compared to unsorted semen (37.1 ± 4.9 % vs. 33.1 ± 6.5 % vs. 7.5 ± 11.6 % and 22.9 ± 7.6 % vs. 21.3 ± 10.3 % vs. 0.0 ± 0.0 % for S-AO-NF vs. S-AO vs. C for 12 and 24 hours respectively). The percentage of spermatozoa with acrosome abnormalities in frozen/thawed samples was significantly higher ($P<0.05$) in S-AO and C group when compared to S-AO-NF (23.0 ± 5.2 %, 22.3 ± 4.0 % and 14.7 ± 7.5 % respectively). The same difference was found for morphological sperm

abnormalities (30.0 ± 6.7 %, 29.0 ± 5.8 % and 19.0 ± 8.1 % respectively). The percentage of acrosome reacted (AR) and viable spermatozoa was analysed by FITC-PNA/PI staining. The percentage of AR spermatozoa was significantly lower ($P<0.001$) in both groups of sorted semen when compared to unsorted control (10.6 ± 3.5 % vs. 6.7 ± 2.6 % vs. 19.1 ± 4.7 % for S-AO, S-AO-NF and C respectively). In addition, the percentage of viable spermatozoa was significantly higher ($P<0.05$) in the S-AO-NF group when compared to groups S-AO and C (72.1 ± 5.0 % vs. 56.9 ± 4.7 % vs. 61.4 ± 8.2 % respectively). Pregnancy rates after artificial insemination with the frozen/thawed semen were not significantly different between sorted spermatozoa and controls (72.7 % vs. 73.3 % vs. 79.2 % for S-AO-NF, S-AO and C respectively).

In conclusion, this study showed that reduction in quality and fertilizing capacity of sex sorted spermatozoa can be successfully prevented by modifications of extenders and handling of the semen before, during and after sperm sorting.

9 Zusammenfassung

Primož Klinc (2005):

Untersuchungen zur Befruchtungskapazität von flowzytometrisch gesextem Bullensperma

Im Rahmen der vorliegenden Untersuchungen wurden der Einfluss der Flowzytometrie sowie die Auswirkung unterschiedlich modifizierter Verdünnerprotokolle auf die Lebensfähigkeit und Befruchtungskapazität von bovinem Frisch- und Tiefgefriersperma analysiert. Die Untersuchung gliedert sich in vier Abschnitte.

Im ersten Teil der Untersuchungen wurden die Auswirkungen der Tiefgefrierung auf Qualität und Befruchtungskapazität flowzytometrisch sortierter Bullenspermien analysiert. Das zu untersuchende Sperma wurde von zwei befruchtungskompetenten Bullen der Rasse „Holstein Friesian“ gewonnen. Die Qualität des Tiefgefrierspermas wurde hinsichtlich Spermienmotilität, Spermienmorphologie sowie der Ergebnisse eines Membranstabilitätstestes (6-CFDA/PI) und eines Kapazitationstestes (FITC-PNA/PI; Induktion mit L-α-Lysophosphatidylcholine –LPC-) ermittelt. Außerdem wurde die Befruchtungsfähigkeit der Spermien im Feldversuch überprüft.

Eine verlängerte Inkubation von stark verdünntem gesextem Sperma vor der Tiefgefrierung hatte einen signifikant ($P<0,05$) negativen Effekt auf die Spermienmotilität und Spermienkapazitation. Positive Auswirkungen auf die Membranstabilität wurden erzielt, wenn Glyzerin erst nach Anpassung auf 5°C kurz vor der Tiefgefrierung zugegeben wurde ($p<0,05$). Glyzerinzugabe bereits während der Äquilibrierungsphase bei Raumtemperatur zeigte dagegen keine Veränderungen Außerdem wurde ein Thermoresistenztest bei 37°C mit gesextem (im Anschluss an Zentrifugation und Glyzerinzugabe bei 5°C) und bei ungesextem Tiefgefriersperma durchgeführt. Unmittelbar nach dem Auftauvorgang unterschied sich die Motilität zwischen gesexten und ungesexten Spermien nicht. Wurden die Spermien jedoch einer weiteren Inkubation bei 37°C unterzogen, verringerte sich deren Motilität jeweils nach drei ($P<0,001$) und sechs ($P<0,05$) Stunden. Gesexte und ungesexte Spermien eines Ejakulates wurden im Feldversuch eingesetzt. Dabei wurden Färsen

und Kühe bei spontaner Brunst oder nach hormoneller Synchronisation durch das Stallpersonal besamt. Wurde der optimale Besamungszeitpunkt durch Brunstbeobachtung ermittelt, führte eine künstliche Besamung mit ungesextem Sperma (56,5 %) zu einer höheren Trächtigkeitsrate (P<0,001) als unter Verwendung von gesextem Sperma (17,6 %). Die Besamung hormonell synchronisierter Tiere hatte keine unterschiedlichen Trächtigkeitsraten zur Folge (36,4 % ungesextes Sperma, 21,3 % gesextes Sperma).

Die Qualität von gesextem, tiefgefrorenem Bullensperma nach Zugabe von Antioxidantien wurde im zweiten Teil dieser Untersuchung verbessert. Von zwei Bullen der Rasse „Holstein Friesian“ wurden insgesamt 12 Ejakulate gewonnen. Jedes dieser Ejakulate wurde in drei Untersuchungsproben aufgeteilt. Zwei Proben wurden auf der Grundlage der „Beltsville Sperm Sexing Technologie“ sortiert, wobei einer Probe jeweils verschiedene Antioxidantien (S-AO) bereits vor dem Sortiervorgang zugesetzt wurden. Eine dritte unsortierte Probe diente als Kontrolle. Alle drei Proben, mit Ausnahme der vor dem Sortiervorgang mit Antioxidantien versetzten Probe, wurden nach dem gleichen Gefrierprotokoll behandelt. Bei Proben, die bereits in Gegenwart der Antioxidantien sortiert wurden, erfolgte die Tiefgefrierung mit einem Antioxidantien enthaltenden TRIS-Eidotter-Verdünner. Zur Bestimmung der Spermienqualität wurden nach dem Auftauvorgang die Spermienmorphologie sofort nach Auftauen der Pailletten und die Spermienmotilität ermittelt. Die Spermienmotilität wurde durch einen Thermoresistenztest nach 0, 6, 12 und 24 Stunden Inkubation bei 37°C analysiert. Außerdem wurden die Spermien mit FITC-PNA/PI fixiert und nach 0, 12 und 24 Stunden Inkubation bei 37°C untersucht. Spermien, die in der Gegenwart von Antioxidantien sortiert wurden (S-AO), waren zu allen Analysezeiten beweglicher (P<0,05) als die ohne Zusätze gesexten Spermien. Sie waren auch nach 0, 6 und 24 Stunden beweglicher als die Spermien der Kontrollgruppe. Der Prozentsatz an Spermien mit geschädigtem Akrosom war in der S-AO-Gruppe (20,8 ± 6,9 %) signifikant geringer (P<0,05) als in der unsortierten Kontrollgruppe (30,3 ± 12,0%). Die S-AO-Gruppe enthielt auch weniger (P<0,05) morphologisch veränderte Spermien (25,8 ± 5,2%) als die unsortierte (36,0 ± 12,5%) und die ohne Antioxidantien sortierte (35,1 ± 7,4%) Gruppe. Keine Unterschiede konnten in der Membranbeschaffenheit der Spermien zwischen den Gruppen festgestellt werden, die im Anschluss und 12 Stunden nach dem Sortiervorgang

mittels FITC/PI fixiert wurden. Nach 24 Stunden Inkubation besaßen die aufgetauten Spermien der S-AO-Gruppe einen signifikant (P<0,001) höheren Prozentsatz (40,7 ± 6,3 %) an unveränderten Membranen im Vergleich zu den beiden anderen Gruppen (unsortiert: 7,8 ± 4,7 %; ohne Antioxidantien sortiert: 7,4 ± 4,6 %). Nach dem Auftauen und einer Inkubation für 0, 12 und 24 Stunden erfolgte die Akrosomreaktion bei prozentual weniger (P<0,05) Spermien der S-AO-Gruppe (14,1 ± 7,5 %; 23,4 ± 5,4 %; 28,8 ± 6,3 %) als bei ungesexten Spermien (25,9 ± 14,4 %; 38,5 ± 16,75 %; 79,8 ± 4,1 %) und herkömmlich gesexten Spermien nach 24 stündiger Inkubation (67,3 ± 10,05). Diese Ergebnisse zeigen erstmals den positiven Effekt von antioxidativen Substanzen auf die Qualität von gesexten und tiefgefrorenen Bullenspermien.

Um die Spermien während des Sortiervorgangs und der anschließenden Tiefgefrierung zu schützen, wurde im dritten Teil dieser Arbeit ein veränderter TRIS-Verdünner zum Einsatz gebracht. Dabei wurde ein speziell für diese Untersuchungen entwickelter Verdünner mit einer spezifischen Kombination an Antioxidantien (AO) und bovinem Serumalbumin (BSA) verwendet. Dieser Verdünner erhöhte die Qualität des gesexten Spermas, und es konnte kein Unterschied weder in der Spermienmotilität noch der Membranbeschaffenheit, die durch FITC-PNA/PI-Färbung ermittelt wurde, zwischen den drei Gruppen beobachtet werden (P>0,05). Im Vergleich zu nicht gesexten Spermien war die Akrosomintegrität in der AO/BSA-Gruppe nach 24 und 48 Stunden Inkubation bei 15°C und in der AO-Gruppe ohne BSA-Zusatz nach 48 stündiger Inkubation besser (P<0,05). Künstlich besamte Färsen nach Brunstbeobachtung wurden durch Sperma aller drei Gruppen in gleichem Maße trächtig (66,7 % ungesexte Spermien; 58,1 % AO/BSA; 54,5 % AO). Außerdem konnten wir erstmalig zeigen, dass sich gesexte Spermien erfolgreich innerhalb von 72 Stunden nach dem Sortiervorgang zur künstlichen Besamung eignen.

Im vierten Teil der Untersuchung wurden die Auswirkungen von Antioxidantien und reversibel inhibierter Spermienmotilität auf die Qualität und Befruchtungskapazität von gesextem Tiefgefriersperma analysiert. Dabei wurden die Spermien sowohl vor als auch nach dem flowzytometrischen Sortiervorgang in ihrer Beweglichkeit gehemmt und ihre Qualität und Befruchtungsfähigkeit ermittelt. Drei verschieden

behandelte Spermiengruppen waren Bestandteil dieser Untersuchung: Gehemmte Spermien, die in Gegenwart von Natriumfluorit (S-AO-NF) sortiert wurden, nach einem Standardprotokoll gesexte Spermien (S-AO) und ungesexte Spermien, die als Kontrolle dienten (C). Im Feldversuch wurden in 197 Betrieben insgesamt 283 Erstbesamungen mit gesextem und ungesextem Tiefgefriersperma von zwei verschiedenen Bullen durchgeführt. Jede Besamungsdosis enthielt 2 Millionen lebende Spermienzellen. Spermien, die mit Natriumfluorit behandelt wurden, waren vollständig unbeweglich, gewannen ihre ursprüngliche Beweglichkeit jedoch nach dem Sortiervorgang und anschließender Zentrifugation wieder zurück. Die Spermienmotilität unterschied sich nach dem Auftauvorgang und anschließender Inkubation bei 37°C nicht zwischen den einzelnen Gruppen. Eine verlängerte Inkubation bei 37°C für 12 und 24 Stunden erhöhte die Spermienmotilität ($P<0,001$) in beiden sortierten Gruppen im Vergleich zur Kontrollgruppe (S-AO-NF: 37,1 ± 4,9 %; 22,9 ± 7,6 % vs. S-AO: 33,1 ± 6,5 %; 21,3 ± 10,3 % vs. C: 7,5 ± 11,6 %; 0,0 ± 0,0 %). Der Prozentsatz an akrosomveränderten Spermien (AR) war höher ($P<0,05$) in der S-AO- und C-Gruppe als in der S-AO-NF-Gruppe (23,0 ± 5,2 %; 22,3 ± 4,0 %; 14,7 ± 7,5 %). Ähnliche Unterschiede konnten für morphologisch veränderte Spermien ermittelt werden (30,0 ± 6,7 %; 29,0 ± 5,8 %; 19,0 ± 8,1 %). Der Prozentsatz an lebenden Spermien, bei denen bereits eine Akrosomreaktion erfolgt war, wurde mittels FITC-PNA/PI-Fixierung analysiert. Dabei hatten prozentual weniger ($P<0,001$) Spermien aus den beiden gesexten Gruppen (S-AO: 10,6 ± 3,5 %; S-AO-NF: 6,7 ± 2,6 %) eine Akrosomreaktion erfahren als Spermien der Kontrollgruppe (19,1 ± 4,7 %). Außerdem konnte in der S-AO-NF-Gruppe (72,1 ± 5,0 %) eine höhere Anzahl lebender Spermien ($P<0,05$) beobachtet werden als in der S-AO- und Kontrollgruppe (56,9 ± 4,7 %; 61,4 ± 8,2 %). Keine Unterschiede gab es in der Trächtigkeitsrate (S-AO-NF: 72,7 %, S-AO: 73,3 %; C: 79,2 %).

Die Ergebnisse dieser Untersuchungen zeigen, dass durch eine veränderte Zusammensetzung des Verdünners und spezielle Behandlungen der Spermien vor, während und nach dem Sortierungsvorgang die Qualität und Befruchtungsfähigkeit von gesexten Spermien erfolgreich an die des ungesexten Spermas angenähert werden können. Bei ausgewählten Bullen sind dementsprechend auch keine speziellen Besamungsverfahren erforderlich.

10 Izvleček

Primož Klinc (2005):

Izboljšanje Oploditvene Sposobnosti Bikovega Semena po Ločevanju Semenčic s Pretočno Citometrijo

V okviru te naloge smo raziskovali vpliv ločevanja semenčic po spolu (sortirano seme) s pomočjo pretočne citometrije ter vpliv prilagoditve oz. spremembe razredčevalcev na kvaliteto in oploditveno sposobnost tekoče konzerviranega in globoko zamrznjenega bikovega semena. Naloga je bila opravljena in razdeljena v štiri med seboj povezane raziskave.

Namen prve raziskave je bil ugotoviti vpliv različnih postopkov priprave sortiranega semena, pred zamrzovanjem v tekočem dušiku, na kvaliteto in oploditveno sposobnost odmrznjenega semena ter ugotavljanje razlik med sortiranim in nesortiranim semenom. Seme je bilo pridobljeno od dveh bikov črno bele pasme. Kvaliteta odmrznjenih vzorcev je bila preiskovana s pomočjo gibljivosti, morfologije, testom za ugotavljanje stabilnosti membran (6-CFDA/PI) ter kapacitacije (barvanje s pomočjo FITC-PNA/PI z dodatkom L-α-Lysophosphatidylcholine). Podaljšana inkubacija visoko razredčenega sortiranega semena je imela statistično značilni negativni vpliv na gibljivost ($P<0,001$) in kapacitacijo ($P<0,05$). Stabilnost celičnih membran je bila statistično značilno ($P<0,001$) višja pri sortiranih vzorcih pri katerih je bil glicerol dodan pri 5°C v primerjavi z dodatkom glicerola pri sobni temperaturi. Neodvisno od postopka priprave je bil ugotovljen v sortiranem v primerjavi z nesortiranim semenom višji odstotek poškodovanih akrosomov in skupnih morfoloških nepravilnosti ($P<0,001$). Poleg osnovnih raziskav je bil opravljen tudi termo-rezistentni test na 37°C. V test je bilo vključeno sortirano (centrifugiranje neposredno po sortiranju in dodatek glicerola pri 5°C) ter nesortirano odmrznjeno seme. Neposredno po tajanju razlika v gibljivosti med sortiranim in nesortiranim semenom ni bila statistično značilna. Po podaljšani inkubaciji na 37°C je bil ugotovljen po 3. ($P<0,001$) in 6. urah ($P<0.05$) statistično značilni nižji odstotek gibljivih semenčic v sortiranem v primerjavi z nesortiranim semenom. Preiskava in primerjava oploditvene sposobnosti sortiranega in nesortiranega semena, je bila opravljena s pomočjo poizkusnih osemenitev na kravah in telicah v času normalnih in

sinhroniziranih pojatev. Pri osemenitvah v normalnih pojatvah je bila ugotovljena statistično značilno višja (P<0,001) brejost z nesortiranim (56,5 %) v primerjavi s sortiranim semenom (17,6 %). Pri živalih s sinhroniziranimi pojatvami je bil odstotek brejih živali osemenjenih z nesortiranim semenom statistično značilno nižji (P= 0,012) v primerjavi z živalmi osemenjenimi v naravnih pojatvah, vendar pa pri tej skupini ni bilo statistično značilne razlike med nesortiranim (36,4 %) in sortiranim semenom (21,3 %).

Namen druge raziskave je bil ugotoviti vpliv antioksidantov na kvaliteto sortiranega in zamrznjenega bikovega semena. Ločevanje semenčic 12. ejakulatov dveh bikov črnobele pasme je bilo opravljeno s pomočjo »Beltsville Sperm Sexing Technology«. Posamezni ejakulat je bil razdeljen na tri dele in obdelan kot nesortirano kontrolno seme, seme sortirano po standardnem postopku ter v prisotnosti antioksidantov (skupina S-AO). Ohlajevanje in zamrzovanje vzorcev je bilo v vseh treh skupinah opravljeno po enakem postopku. Edina razlika je bila dodatek anitoksidantov v razredčevalce za zamrzovanje v skupini, ki je bila sortirana v prisotnosti antioksidantov. Nadzor kvalitete semena po odmrzovanju je bilo opravljeno s pomočjo analize morfologije, ugotavljanje gibljivosti semenčic v 24 urnem termo-rezistenčnem testu (37°C) ter ugotavljanje vitalnosti semenčic s pomočjo FITC-PNA/PI preiskave po 0, 12 in 24 urni inkubaciji na 37°C. Gibljivost semenčic je bila 0, 6 in 24 ur po tajanju statistično značilno višja (P<0,05) v S-AO skupini v primerjavi z nesortiranim semenom ter statistično značilno višja (P<0,05) v primerjavi s standardno sortiranim semenom v vseh preiskovanih časovnih intervalih. Odstotek semenčic s poškodovanimi akrosomi je bil statistično značilno nižji (P<0,05) v S-AO vzorcih v primerjavi z nesortiranim semenom (20,8 ± 6,9 % proti 30,3 ± 12,0 %). Odstotek semenčic z morfološkimi spremembami (P<0,05) je bil statistično značilno nižji v S-AO skupini v primerjavi z nesortiranim in semenom sortiranim po standardnem postopku (25,8 ± 5,2 %; 36,0 ± 12,5 % in 35,1 ± 7,4 %). Preiskava vitalnosti semenčic s pomočjo FITC-PNA/PI ni pokazala statistično značilne razlike med skupinami 0 in 12 ur po tajanju, po 24 urni inkubaciji pa je bil odstotek semenčic z nepoškodovanimi membranami statistično značilno višji (P<0,001) v S-AO skupini v primerjavi z nesortiranim in semenom sortiranim po standardnem postopku (40,7 ± 6,3 %; 7,8 ± 4,7 % in 7,4 ± 4,6 %). Odstotek semenčic z reagiranimi akrosomi je bil statistično značilno nižji (P<0,05) v S-AO skupini v primerjavi z nesortiranim

semenom 0, 12 in 24 ur po tajanju (14,1 ± 7,5 %, 23,4 ± 5,4 % in 28,8 ± 6,3 % proti 25,9 ± 14,4 %, 38,5 ± 16,7 % in 79,8 ± 4,1 %, za 0, 12, 24 ur ter S-AO proti nesortirano seme) ter v primerjavi s semenom sortiranim po standardnem postopku 24 ur po tajanju (28,8 ± 6,3 % proti 67,3 ± 10,0 %). Ta raziskava je prvič pokazala visok pozitivni vpliv antioksidantov na kvaliteto sortiranega in globoko zamrznjenega bikovega semena.

V tretjo raziskavo so bile vključene spremembe postopka z namenom zaščite semenčic v času ločevanja s pomočjo pretočne citometrije in tekočega konzerviranja. Posebno pripravljena kombinacija antioksidantov (AO) in govejega albuminskega seruma (BSA) je namenjena podaljšanju vitalnosti semenčic. Z uporabo novega razredčevalca je bila gibljivost sortiranih semenčic primerljiva z gibljivostjo v nesortiranem semenu. Integriteta celičnih membran je bila preiskovana s pomočjo FITC-PNA/PI in se prav tako ni razlikovala od nesortiranega semena. Integriteta akrosomov je bila statistično značilno višja pri sortiranem semenu v AO/BSA skupini po 24 in 48 urni inkubaciji na 15°C ($P<0,05$) in prav tako po 48 urni inkubaciji v AO skupini ($P<0,05$) v primerjavi z nesortiranim semenom. Poleg tega ni bilo statistično značilne razlike v odstotku brejih živali, ki so bile osemenjene z nesortiranim (66.7 %) ter sortiranim semenom iz skupine AO/BSA (58,1 %) in skupine AO (54,5 %). V tej raziskavi je bilo tudi prvič ugotovljeno podaljšanje oploditvene sposobnosti sortiranega in tekoče konzerviranega semena iz nekaj ur na 72 ur po sortiranju.

Namen četrte raziskave je bil preučevanje vpliva antioksidantov in začasne kemične preprečitve gibljivosti semenčic pred in v času pretočne citometrije na vitalnost in oploditveno sposobnost sortiranega in globoko zamrznjenega semena. Vitalnost in oploditvena sposobnost semenčic po začasni preprečitvi gibljivosti z natrijevim fluoridom (skupina S-AO-NF) je bila primerjana s semenom seksiranim po standardnem postopku razvitim v našem laboratoriju (S-AO) ter nesortiranim kontrolnim semenom (C). V raziskavo je bilo vključeno seme dveh bikov (limuzin in črno bela pasma) ter skupno 283 prvih osemenitev z dvema milijonoma vitalnih sortiranih ter nesortiranih semenčic po odmrzovanju. Gibljivost semenčic je bila povsem preprečena po dodatku natrijevega fluorida, vendar se je po ločevanju z pretočno citometrijo in centrifugiranju vrnila na predhodno vrednost. Po odmrzovanju in inkubaciji semena na 37°C ni bilo razlike med posameznimi skupinami. Po

podaljšanju inkubacije semena na 37°C za 12 in 24 ur je bila gibljivost v obeh skupinah sortiranega semena statistično značilno višja (P<0,001) v primerjavi z nesortiranim semenom (37,1 ± 4,9 % proti 33,1 ± 6,5 % proti 7,5 ± 11,6 % in 22,9 ± 7,6 % proti 21,3 ± 10,3 % proti 0,0 ± 0,0 % za S-AO-NF proti S-AO proti C za 12 in 24 ur). V odmrznjenih vzorcih je bil odstotek semenčic s poškodovanimi akrosomi statistično značilno višji (P<0,05) v S-AO in C skupini v primerjavi s S-AO-NF skupino (23,0 ± 5,2 %, 22,3 ± 4,0 % in 14,7 ± 7,5 % za S-AO, C in S-AO-NF skupino). Enaka statistično značilna razlika je bila ugotovljena tudi za skupne morfološke napake (30,0 ± 6,7 %, 29,0 ± 5,8 % in 19,0 ± 8,1 % za S-AO, C in S-AO-NF skupino). Odstotek semenčic z reagiranimi akrosomi (RA) in njihova vitalnost sta bila preučevana s FITC-PNA/PI preiskavo. Odstotek semenčic z RA je bil statistično značilno nižji (P<0.001) v obeh skupinah sortiranega semena v primerjavi z nesortiranim semenom (10,6 ± 3,5 % proti 6,7 ± 2,6 % proti 19,1 ± 4,7 % za S-AO proti S-AO-NF proti C). Odstotek vitalnih semenčic pa je bil statistično značilno višji (P<0.05) v S-AO-NF skupini v primerjavi s S-AO in C skupino (72,1 ± 5,0 % proti 56,9 ± 4,7 % proti 61,4 ± 8,2 % za S-AO-NF proti S-AO proti C). V dodatni preiskavi plodnosti ni bilo ugotovljene statistično značilne razlike v odstotku brejih živali osemenjenih z sortiranim ali nesortiranim semenom (72,7 % proti 73,3 % proti 79,2% za S-AO-NF proti S-AO proti C).

Rezultati te raziskave so pokazali, da je zmanjšanje vitalnosti in oploditvene sposobnosti sortiranih semenčic mogoče uspešno preprečiti s prilagoditvijo razredčevalcev in postopkov pred, med in po ločevanju semenčic s pretočno citometrijo.

11 References

ABEYDEERA, L. R., L. A. JOHNSON, G. R. WELCH, W. H. WANG, A. C. BOQUEST, T. C. CANTLEY, A. RIEKE and B. N. DAY (1998):
Birth of piglets preselected for gender following in vitro fertilization of in vitro matured pig oocytes by X and Y chromosome bearing spermatozoa sorted by high speed flow cytometry.
Theriogenology 50, 981-988

ALI, J. I., F. E. ELDRIDGE, G. C. KOO and B. D. SCHANBACHER (1990):
Enrichment of bovine X- and Y-chromosome-bearing sperm with monoclonal H-Y antibody-fluorescence-activated cell sorter.
Arch. Androl 24, 235-245

ALVAREZ, J. G., and B. T. STOREY (1985):
Spontaneous lipid peroxidation in rabbit and mouse epididymal spermatozoa: dependence of rate on temperature and oxygen concentration.
Biol. Reprod. 32, 342-351

ANDERSSON, M., J. TAPONEN, E. KOSKINEN and M. DAHLBOM (2004):
Effect of insemination with doses of 2 or 15 million frozen-thawed spermatozoa and semen deposition site on pregnancy rate in dairy cows.
Theriogenology 61, 1583-1588

ASKARI, H. A., J. H. CHECK, N. PEYMER and A. BOLLENDORF (1994):
Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process.
Arch. Androl 33, 11-15

BALDI, E., M. LUCONI, L. BONACCORSI, C. KRAUSZ and G. FORTI (1996):
Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodelling pathways.
Front Biosci. 1, 189-205

BARRIOS, B., R. PEREZ-PE, M. GALLEGO, A. TATO, J. OSADA, T. MUINO-BLANCO and J. A. CEBRIAN-PEREZ (2000):
Seminal plasma proteins revert the cold-shock damage on ram sperm membrane.
Biol. Reprod. 63, 1531-1537

BAUMBER, J., B. A. BALL, C. G. GRAVANCE, V. MEDINA and M. C. DAVIES-MOREL (2000):
The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation.
J Androl 21, 895-902

BEAL, W. E., L. M. WHITE and D. L. GARNER (1984):
Sex ratio after insemination of bovine spermatozoa isolated using a bovine serum albumin gradient.
J Anim Sci 58, 1432-1436

BECONI, M. T., C. R. FRANCIA, N. G. MORA and M. A. AFFRANCHINO (1993):
Effect of natural antioxidants on frozen bovine semen preservation.
Theriogenology 40, 841-851

BEERNINK, F. J., and R. J. ERICSSON (1982):
Male sex preselection through sperm isolation.
Fertil. Steril. 38, 493-495

BHATTACHARYA, B. C. (1962):
Different sedimentation rates of X- and Y- sperm and the question of arbitrary sex determination.
Z. Wiss. Zool. 166, 203-250

BHATTACHARYA, B. C., A. D. BANGHAM, R. J. CRO, R. D. KEYNES and L. E. ROWSON (1966):
An attempt to predetermine the sex of calves by artificial insemination with spermatozoa separated by sedimentation.
Nature 211, 863-

BILODEAU, J. F., S. BLANCHETTE, N. CORMIER and M. A. SIRARD (2002):
Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase.
Theriogenology 57, 1105-1122

BILODEAU, J. F., S. CHATTERJEE, M. A. SIRARD and C. GAGNON (2000):
Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing.
Mol. Reprod. Dev. 55, 282-288

BLOTTNER, S., H. BOSTEDT, K. MEWES and C. PITRA (1994):
Enrichment of bovine X and Y spermatozoa by free-flow electrophoresis.
Zentralbl. Veterinarmed. A 41, 466-474

BONE, W., N. G. JONES, G. KAMP, C. H. YEUNG and T. G. COOPER (2000):
Effect of ornidazole on fertility of male rats: inhibition of a glycolysis-related motility pattern and zona binding required for fertilization in vitro.
J Reprod. Fertil. 118, 127-135

BREITBART, H., T. LEVINSHAL, N. COHEN, H. FRIEDMANN and R. LUBART (1996):
Changes in calcium transport in mammalian sperm mitochondria and plasma membrane irradiated at 633 nm (HeNe laser).
J Photochem. Photobiol. B 34, 117-121

BROWN-WOODMAN, P. D., and I. G. WHITE (1976):
A comparison of the inhibition of the metabolism of ram and rabbit spermatozoa be alpha-chlorohydrin in vitro.
Theriogenology 6, 29-37

BUCHANAN, B. R., G. E. SEIDEL, JR., P. M. MCCUE, J. L. SCHENK, L. A. HERICKHOFF and E. L. SQUIRES (2000):
Insemination of mares with low numbers of either unsexed or sexed spermatozoa.
Theriogenology 53, 1333-1344

CALAMERA, J. C., P. J. FERNANDEZ, M. G. BUFFONE, A. A. ACOSTA and G. F. DONCEL (2001):
Effects of long-term in vitro incubation of human spermatozoa: functional parameters and catalase effect.
Andrologia 33, 79-86

CATT, S. L., J. W. CATT, M. C. GOMEZ, W. M. MAXWELL and G. EVANS (1996):
Birth of a male lamb derived from an in vitro matured oocyte fertilised by intracytoplasmic injection of a single presumptive male sperm.
Vet Rec 139, 494-495

CATT, S. L., D. SAKKAS, D. BIZZARO, P. G. BIANCHI, W. M. MAXWELL and G. EVANS (1997):
Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human spermatozoa.
Mol. Hum. Reprod. 3, 821-825

CHATTERJEE, S. and C. GAGNON (2001):
Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing.
Mol. Reprod. Dev. 59, 451-458

CHINOY, N. J. and M. V. NARAYANA (1994):
In vitro fluoride toxicity in human spermatozoa.
Reprod. Toxicol. 8, 155-159

CONAWAY, C. C., J. WHYSNER, L. K. VERNA and G. M. WILLIAMS (1996):
Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation.
Pharmacol. Ther. 71, 29-55

COONEY, S. J. and A. R. JONES (1988):
Inhibitory effects of (S)-3-chlorolactaldehyde on the metabolic activity of boar spermatozoa in vitro.
J Reprod. Fertil. 82, 309-317

CORIAT, A. M., E. VALLELEY, M. W. FERGUSON and P. T. SHARPE (1994):
Chromosomal and temperature-dependent sex determination: the search for a conserved mechanism.
J Exp. Zool. 270, 112-116

CRAN, D. G., D. J. COCHRANE, D. J. JOHNSON, H. WEI, K. H. LU, AND C. POLGE (1994):
Separation of X- and Y-chromosome bearing bovine sperm by flow cytometry for use in IVF.
Theriogenology 41, 183-183

CRAN, D. G., L. A. JOHNSON, N. G. MILLER, D. COCHRANE and C. POLGE (1993):
Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertilisation.
Vet Rec 132, 40-41

CRAN, D. G., W. A. C. MCKELVEY, M. E. KING, D. F. DOLMAN, T. G. MCEVOY, P. J. BROADBENT, AND J. J. ROBINSON (1997):
Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted sperm.
Theriogenology 47, 267

CRITSER, J. K., A. R. HUSE-BENDA, D. V. AAKER, B. W. ARNESON and G. D. BALL (1988):
Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility.
Fertil. Steril. 50, 314-320

DALTON, J. C., S. NADIR, J. H. BAME, M. NOFTSINGER and R. G. SAACKE (2000):
The effect of time of artificial insemination on fertilization status and embryo quality in superovulated cows.
J. Anim Sci. 78, 2081-2085

DE LAMIRANDE, E., and C. GAGNON (1992):
Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes.
J Androl 13, 368-378

DEAN, P. N., D. PINKEL and M. L. MENDELSOHN (1978):
Hydrodynamic orientation of sperm heads for flow cytometry.
Biophys. J 23, 7-13

DEJARNETTE, J. M., R. G. SAACKE, J. BAME and C. J. VOGLER (1992):
Accessory sperm: their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle.
J. Anim Sci. 70, 484-491

DMOWSKI, W. P., L. GAYNOR, R. RAO, M. LAWRENCE and A. SCOMMEGNA (1979):
Use of albumin gradients for X and Y sperm separation and clinical experience with male sex preselection.
Fertil. Steril. 31, 52-57

DORIZZI, M., N. RICHARD-MERCIER and C. PIEAU (1996):
The ovary retains male potential after the thermosensitive period for sex determination in the turtle Emys orbicularis.
Differentiation 60, 193-201

DOTT, H. M., R. M. MOOR and C. POLGE (1976):
Artificial insemination with spermatozoa in formaldehyde.
J Reprod. Fertil. 46, 277

DOYLE, S. P., G. E. SEIDEL, J. L. SCHENK, L. A. HERICKHOFF, D. G. CRAN and R. D. GREEN (1999):
Artificial insemination of lactating Angus cows with sexed semen.
Proc. Western Section Am. Soc. Anim. Sci. 50, 203-205

ENGELMANN, and, F. KRASSNIGG, H. SCHATZ and W. B. SCHILL (1988):
Separation of human X and Y spermatozoa by free-flow electrophoresis.
Gamete Res. 19, 151-160

ERBA, E., P. UBEZIO, M. BROGGINI, M. PONTI and M. D'INCALCI (1988):
DNA damage, cytotoxic effect and cell-cycle perturbation of Hoechst 33342 on L1210 cells in vitro.
Cytometry 9, 1-6

ERICKSON, R. P., S. E. LEWIS and M. BUTLEY (1981):
Is Haploid gene expression possible for sperm antigens?
J Reprod. Immunol. 3, 195-217

ERICSSON, R. J., C. N. LANGEVIN and M. NISHINO (1973):
Isolation of fractions rich in human Y sperm.
Nature 246, 421-424

ERRASFA, M., and A. STERN (1994):
Inhibition of epidermal growth factor-dependent protein tyrosine phosphorylation by phorbol myristate acetate is mediated by protein tyrosine phosphatase activity.
FEBS Lett. 339, 7-10

FLAHERTY, S. P., J. MICHALOWSKA, N. J. SWANN, W. P. DMOWSKI, C. D. MATTHEWS and R. J. AITKEN (1997):
Albumin gradients do not enrich Y-bearing human spermatozoa.
Hum. Reprod. 12, 938-942

FLINT, A. F., P. L. CHAPMAN and G. E. SEIDEL, JR. (2003):
Fertility assessment through heterospermic insemination of flow-sorted sperm in cattle.
J. Anim Sci. 81, 1814-1822

FOOTE, R. H. (2001):
Inhibition of sperm motility does not affect live-dead separation of bull sperm by glass beads.
Asian J Androl 3, 193-198

FUGGER, E. F. (1999):
Clinical experience with flow cytometric separation of human X- and Y-chromosome bearing sperm.
Theriogenology 52, 1435-1440

FULWYLER, M. J. (1977):
Hydrodynamic orientation of cells.
J Histochem. Cytochem. 25, 781-783

GABRIEL, W. N., B. BLUMBERG, S. SUTTON, A. R. PLACE and V. A. LANCE (2001):
Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures.
J Exp. Zool. 290, 439-448

GARNER, D. L., B. L. GLEDHILL, D. PINKEL, S. LAKE, D. STEPHENSON, M. A. VAN DILLA and L. A. JOHNSON (1983):
Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry.
Biol. Reprod. 28, 312-321

GARNER, D. L., D. PINKEL, L. A. JOHNSON and M. M. PACE (1986):
Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses.
Biol. Reprod. 34, 127-138

GARNER, D. L., C. A. THOMAS, C. G. GRAVANCE, C. E. MARSHALL, J. M. DEJARNETTE and C. H. ALLEN (2001):
Seminal plasma addition attenuates the dilution effect in bovine sperm.
Theriogenology 56, 31-40

GIL, J., N. LUNDEHEIM, L. SODERQUIST and H. RODRIIUEZ-MARTINEZ (2003):
Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen.
Theriogenology 59, 1241-1255

GLEDHILL, B. L., S. LAKE, L. L. STEINMETZ, J. W. GRAY, CRAWFORD, P. N. DEAN and M. A. VAN DILLA (1976):
Flow microfluorometric analysis of sperm DNA content: effect of cell shape on the fluorescence distribution.
J Cell Physiol 87, 367-375

GROSSFELD, R., P. KLINC, B. SIEG, AND D. RATH (2005):
Production of piglets with sexed semen employing a non-surgical insemination technique.
Theriogenology (im Druck)

HANCOCK, R. J. (1978):
Comparison of effects of normal rabbit sera and anti-cock sperm sera on rabbit sperm, including comparison of effects on the sex ratio.
Biol. Reprod. 18, 510-515

HARLEY, V. R., D. I. JACKSON, P. J. HEXTALL, J. R. HAWKINS, G. D. BERKOVITZ, S. SOCKANATHAN, R. LOVELL-BADGE and P. N. GOODFELLOW (1992):
DNA binding activity of recombinant SRY from normal males and XY females.
Science 255, 453-456

HARRISON, R. A., and S. E. VICKERS (1990):
Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa.
J Reprod. Fertil. 88, 343-352

HENDRIKSEN, P. J., M. TIEMAN, L. T. VAN DER and L. A. JOHNSON (1993):
Binding of anti-H-Y monoclonal antibodies to separated X and Y chromosome-bearing porcine and bovine sperm.
Mol. Reprod. Dev. 35, 189-196

HOLLINSHEAD, F. K., G. EVANS, K. M. EVANS, S. L. CATT, W. M. MAXWELL and J. K. O'BRIEN (2004):
Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa.
Reproduction. 127, 557-568

HOLLINSHEAD, F. K., L. GILLAN, J. K. O'BRIEN, G. EVANS and W. M. MAXWELL (2003):
In vitro and in vivo assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing.
Reprod. Fertil. Dev. 15, 351-359

HOLLINSHEAD, F. K., J. K. O'BRIEN, L. HE, W. M. C. MAXWELL, AND G. EVANS (2001):
Pregnancies after insemination of ewes with sorted, cryopreserved ram spermatozoa.
Proc.Soc.Reprod.Biol. 32, 20

HOLLINSHEAD, F. K., J. K. O'BRIEN, W. M. MAXWELL and G. EVANS (2002):
Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen-thawed sorted X- or Y-chromosome-bearing spermatozoa.
Reprod. Fertil. Dev. 14, 503-508

HUNTER, R. H. F. (1995):
Sex determinatin, differentiation and intersexuality in placental mammals.
Cambridge University Press

ICHIKAWA, T., T. OEDA, H. OHMORI and W. B. SCHILL (1999):
Reactive oxygen species influence the acrosome reaction but not acrosin activity in human spermatozoa.
Int. J Androl 22, 37-42

ISHIKAWA, S., Y. YANO, K. ARIHARA and M. ITOH (2004):
Egg yolk phosvitin inhibits hydroxyl radical formation from the fenton reaction.
Biosci. Biotechnol. Biochem. 68, 1324-1331

JACOBS, P. A., and J. A. STRONG (1959):
A case of human intersexuality having a possible XXY sex-determining mechanism.
Nature 183, 302-303

JAGER, R. J., M. ANVRET, K. HALL and G. SCHERER (1990):
A human XY female with a frame shift mutation in the candidate testis-determining gene SRY.
Nature 348, 452-454

JANUSKAUSKAS, A., L. SODERQUIST, M. G. HAARD, M. C. HAARD, N. LUNDEHEIM and H. RODRIGUEZ-MARTINEZ (1996):
Influence of sperm number per straw on the post-thaw sperm viability and fertility of Swedish red and white A.I. bulls.
Acta Vet. Scand. 37, 461-470

JOHNSON, L. A. (1991):
Sex preselection in swine: Altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm.
Reprod. Dom. Anim. 26, 309-314

JOHNSON, L. A., and R. N. CLARKE (1988):
Flow sorting of X and Y chromosome-bearing mammalian sperm: activation and pronuclear development of sorted bull, boar, and ram sperm microinjected into hamster oocytes.
Gamete Res. 21, 335-343

JOHNSON, L. A., J. P. FLOOK and H. W. HAWK (1989):
Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting.
Biol. Reprod. 41, 199-203

JOHNSON, L. A., J. P. FLOOK and M. V. LOOK (1987a):
Flow cytometry of X and Y chromosome-bearing sperm for DNA using an improved preparation method and staining with Hoechst 33342.
Gamete Res. 17, 203-212

JOHNSON, L. A., J. P. FLOOK, M. V. LOOK and D. PINKEL (1987b):
Flow sorting of X and Y chromosome-bearing spermatozoa into two populations.
Gamete Res. 16, 1-9

JOHNSON, L. A., H. D. GUTHRIE, P. FISHER, W. M. C. MAXWELL, WELCH G.R., and GERRETT.W.M. (2000):
Cryopreservation of flow cytometrically sorted boar sperm: effects on in vivo embryo development.
J Anim Sci 78 (suppl. 1), 198

JOHNSON, L. A., and D. PINKEL (1986):
Modification of a laser-based flow cytometer for high-resolution DNA analysis of mammalian spermatozoa.
Cytometry 7, 268-273

JOHNSON, L. A., G. R. WELCH and W. RENS (1999):
The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI.
J. Anim Sci. 77 Suppl 2, 213-220

KANEKO, S., J. YAMAGUCHI, T. KOBAYASHI and R. IIZUKA (1983):
Separation of human X- and Y-bearing sperm using percoll density gradient centrifugation.
Fertil. Steril. 40, 661-665

KAWARASAKI, T., G. R. WELCH, C. R. LONG, M. YOSHIDA and L. A. JOHNSON (1998):
Verification of flow cytometorically-sorted X- and Y-bearing porcine spermatozoa and reanalysis of spermatozoa for DNA content using the fluorescence in situ hybridization (FISH) technique.
Theriogenology 50, 625-635

KIM, J. G., and S. PARTHASARATHY (1998):
Oxidation and the spermatozoa.
Semin. Reprod. Endocrinol. 16, 235-239

KNÖPPEL, S. (2001):
Lektinbindungsvermögen von gesextem und ungesextem Sperma.
Soest, GHS Paderborn, Diplomarbeit

KOBAYASHI, J., H. OGURO, H. UCHIDA, T. KOHSAKA, H. SASADA and E. SATO (2004):
Assessment of bovine X- and Y-bearing spermatozoa in fractions by discontinuous percoll gradients with rapid fluorescence in situ hybridization.
J Reprod. Dev. 50, 463-469

KODAMA, H., Y. KURIBAYASHI and C. GAGNON (1996):
Effect of sperm lipid peroxidation on fertilization.
J Androl 17, 151-157

KOOPMAN, P., J. GUBBAY, N. VIVIAN, P. GOODFELLOW and R. LOVELL-BADGE (1991):
Male development of chromosomally female mice transgenic for Sry.
Nature 351, 117-121

KOOPMAN, P., A. MUNSTERBERG, B. CAPEL, N. VIVIAN and R. LOVELL-BADGE (1990):
Expression of a candidate sex-determining gene during mouse testis differentiation.
Nature 348, 450-452

LEVINSON, G., K. KEYVANFAR, J. C. WU, E. F. FUGGER, R. A. FIELDS, G. L. HARTON, F. T. PALMER, M. E. SISSON, K. M. STARR and L. DENNISON-LAGOS (1995):
DNA-based X-enriched sperm separation as an adjunct to preimplantation genetic testing for the prevention of X-linked disease.
Hum. Reprod. 10, 979-982

LINDSEY, A. C., L. H. MORRIS, W. R. ALLEN, J. L. SCHENK, E. L. SQUIRES and J. E. BRUEMMER (2002):
Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa.
Equine Vet J 34, 128-132

LOPEZ, O., A. MATA, M. ANTICH and L. BASSAS (1993):
Sperm selection by PD-10 sephadex columns: comparison with SpermPrep filtration and Percoll centrifugation.
Hum. Reprod. 8, 732-736

LU, C. L., and R. C. BAKER (1986):
Characteristics of egg yolk phosvitin as an antioxidant for inhibiting metal-catalyzed phospholipid oxidations.
Poult. Sci 65, 2065-2070

LU, K. H., D. G. CRAN and G. E. SEIDEL, JR. (1999):
In vitro fertilization with flow-cytometrically-sorted bovine sperm.
Theriogenology 52, 1393-1405

LUBART, R., H. FRIEDMANN, T. LEVINSHAL, R. LAVIE and H. BREITBART (1992):
Effect of light on calcium transport in bull sperm cells.
J Photochem. Photobiol. B 15, 337-341

LUBART, R., H. FRIEDMANN, M. SINYAKOV, N. COHEN and H. BREITBART (1997):
Changes in calcium transport in mammalian sperm mitochondria and plasma membranes caused by 780 nm irradiation.
Lasers Surg. Med. 21, 493-499

LUCONI, M., I. PORAZZI, P. FERRUZZI, S. MARCHIANI, G. FORTI and E. BALDI (2005):
Tyrosine Phosphorylation of the A Kinase Anchoring Protein 3 (AKAP3) and Soluble Adenylate Cyclase Are Involved in the Increase of Human Sperm Motility by Bicarbonate.
Biol. Reprod. 72, 22-32

MANGER, M., H. BOSTEDT, W. B. SCHILL and A. J. MILEHAM (1997):
Effect of sperm motility on separation of bovine X- and Y-bearing spermatozoa by means of free-flow electrophoresis.
Andrologia 29, 9-15

MAXWELL, W. M., G. EVANS, F. K. HOLLINSHEAD, R. BATHGATE, S. P. DE GRAAF, B. M. ERIKSSON, L. GILLAN, K. M. MORTON and J. K. O'BRIEN (2004):
Integration of sperm sexing technology into the ART toolbox.
Anim Reprod. Sci 82-83, 79-95

MAXWELL, W. M., and L. A. JOHNSON (1999):
Physiology of spermatozoa at high dilution rates: the influence of seminal plasma.
Theriogenology 52, 1353-1362

MAXWELL, W. M., C. R. LONG, L. A. JOHNSON, J. R. DOBRINSKY and G. R. WELCH (1998):
The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma.
Reprod. Fertil. Dev. 10, 433-440

MAXWELL, W. M., and T. STOJANOV (1996):
Liquid storage of ram semen in the absence or presence of some antioxidants.
Reprod. Fertil. Dev. 8, 1013-1020

MAXWELL, W. M., G. R. WELCH and L. A. JOHNSON (1996):
Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma.
Reprod. Fertil. Dev. 8, 1165-1178

MCLAREN, A., and M. MONK (1981):
X-chromosome activity in the germ cells of sex-reversed mouse embryos.
J Reprod. Fertil. 63, 533-537

MCNUTT, T. L., and G. J. KILLIAN (1991):
Influence of bovine follicular and oviduct fluids on sperm capacitation in vitro.
J Androl 12, 244-252

MONTAG, M., K. RINK, G. DELACRETAZ and D. VAN, V (2000):
Laser-induced immobilization and plasma membrane permeabilization in human spermatozoa.
Hum. Reprod. 15, 846-852

MORRELL, J. M., K. D. KEELER, D. E. NOAKES, N. M. MACKENZIE and D. W. DRESSER (1988):
Sexing of sperm by flow cytometry.
Vet Rec 122, 322-324

MORTON, K. M., S. L. CATT, F. K. HOLLINSHEAD, W. M. C. MAXWELL and G. EVANS (2004):
Lambs born after in vitro embryo production from prepubertal lamb oocytes and frozen-thawed unsorted and sex-sorted spermatozoa.
Reprod. Fertil. Dev. 16, 260-260

MORUZZI, J. F. (1979):
Selecting a mammalian species for the separation of X- and Y-chromosome-bearing spermatozoa.
J Reprod. Fertil. 57, 319-323

MOUSSA, M., V. MARINET, A. TRIMECHE, D. TAINTURIER and M. ANTON (2002):
Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen.
Theriogenology 57, 1695-1706

MULLER, and, T. DONLON, M. SCHMID, N. FITCH, C. L. RICHER, M. LALANDE and S. A. LATT (1986):
Deletion mapping of the testis determining locus with DNA probes in 46,XX males and in 46,XY and 46,X,dic(Y) females.
Nucleic Acids Res. 14, 6489-6505

NADIR, S., R. G. SAACKE, J. BAME, J. MULLINS and S. DEGELOS (1993):
Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle.
J. Anim Sci. 71, 199-204

OEHNINGER, S., P. BLACKMORE, M. MAHONY and G. HODGEN (1995):
Effects of hydrogen peroxide on human spermatozoa.
J Assist. Reprod. Genet. 12, 41-47

PAGE, D. C., R. MOSHER, E. M. SIMPSON, E. M. FISHER, G. MARDON, J. POLLACK, B. MCGILLIVRAY, C. A. DE LA and L. G. BROWN (1987):
The sex-determining region of the human Y chromosome encodes a finger protein.
Cell 51, 1091-1104

PALMER, M. S., A. H. SINCLAIR, P. BERTA, N. A. ELLIS, P. N. GOODFELLOW, N. E. ABBAS and M. FELLOUS (1989):
Genetic evidence that ZFY is not the testis-determining factor.
Nature 342, 937-939

PARRILLA, I., J. M. VAZQUEZ, C. CUELLO, M. A. GIL, J. ROCA, D. DI BERARDINO and E. A. MARTINEZ (2004):
Hoechst 33342 stain and andv. laser exposure do not induce genotoxic effects in flow-sorted boar spermatozoa.
Reproduction. 128, 615-621

PENA, F. J., A. JOHANNISSON, M. WALLGREN and M. H. RODRIGUEZ (2003):
Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate.
Anim Reprod. Sci 78, 85-98

PETERSON, R. N., and M. FREUND (1977):
Reversible inhibition of the motility of human spermatozoa by cytochalasin B.
Fertil. Steril. 28, 257-261

PICKETT, B. W., and R. P. AMANN (1993):
Cryopreservation of semen.
in: Equine reproduction. S. 769-789

PIEAU, C., M. DORIZZI and N. RICHARD-MERCIER (2001):
Temperature-dependent sex determination and gonadal differentiation in reptiles.
EXS 117-141

PINKEL, D., S. LAKE, B. L. GLEDHILL, M. A. VAN DILLA, D. STEPHENSON and G. WATCHMAKER (1982):
High resolution DNA content measurements of mammalian sperm.
Cytometry 3, 1-9

PORTER, L. M., and A. R. JONES (1995):
Inhibition of glyceraldehyde 3-phosphate dehydrogenase in boar spermatozoa by bromohydroxypropanone.
Reprod. Fertil. Dev. 7, 107-111

POSO, H., K. WICHMANN, J. JANNE and T. LUUKKAINEN (1980):
Gossypol, a powerful inhibitor of human spermatozoal metabolism.
Lancet 1, 885-886

PRESICCE, G. A., S. VERBERCKMOES, E. M. SENATORE, P. KLINC and D. RATH (2005):
First established pregnancies in Mediterranean Italian buffaloes (Bubalus bubalis) following deposition of sexed spermatozoa near the utero tubal junction.
Reprod. Domest. Anim 40, 73-75

PROBST, S., and D. RATH (2003):
Production of piglets using intracytoplasmic sperm injection (ICSI) with flowcytometrically sorted boar semen and artificially activated oocytes.
Theriogenology 59, 961-973

PURSLEY, J. R., M. C. WILTBANK, J. S. STEVENSON, J. S. OTTOBRE, H. A. GARVERICK and L. L. ANDERSON (1997):
Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus.
J. Dairy Sci. 80, 295-300

PYRZAK, R. (1994):
Separation of X- and Y-bearing human spermatozoa using albumin gradients.
Hum. Reprod. 9, 1788-1790

QUINLIVAN, W. L., K. PRECIADO, T. L. LONG and H. SULLIVAN (1982):
Separation of human X and Y spermatozoa by albumin gradients and Sephadex chromatography.
Fertil. Steril. 37, 104-107

RATH, D., L. A. JOHNSON, J. R. DOBRINSKY, WELCH G.R. and NIEMANN H. (1997):
Production of piglets preselected for sex following in vitro fertilization with X and Y chromosome-bearing spermatozoa sorted by flow cytometry.
Theriogenology 47, 795-800

RATH, D., L. A. JOHNSON, AND WELCH G.R. (1993):
In vitro culture of porcine embryos: Development to blastocysts after in vitro fertilization (IVF) with flow cytometrically sorted and unsorted semen.
Theriogenology 39, 293-293

RATH, D., C. R. LONG, J. R. DOBRINSKY, G. R. WELCH, L. L. SCHREIER and L. A. JOHNSON (1999):
In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer.
J Anim Sci 77, 3346-3352

RATH, D., S. RUIZ and B. SIEG (2003a):
Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen.
Vet Rec 152, 400-401

RATH, D., B. SIEG, J. LEIGH, P. KLINC, M. BESSELING, C. KRÜGER, WOLKEN, A, A. FRENZEL, P. WESTERMANN, S. PROBST, R. GROSSFELD, K. G. HADLER and C. EHLING (2003b):
Current perspectives of sperm sorting in domestic farm animals. In: proceeeding of the 19th meeting Association Europenne de Transferet Embryonnaire.
Rostock, Germany 125-128

RENS, W., G. R. WELCH and L. A. JOHNSON (1998):
A novel nozzle for more efficient sperm orientation to improve sorting efficiency of X and Y chromosome-bearing sperm.
Cytometry 33, 476-481

RIVLIN, J., J. MENDEL, S. RUBINSTEIN, N. ETKOVITZ and H. BREITBART (2004):
Role of hydrogen peroxide in sperm capacitation and acrosome reaction.
Biol. Reprod. 70, 518-522

ROHDE, W., T. PORSTMANN, S. PREHN and G. DORNER (1975):
Gravitational pattern of the Y-bearing human spermatozoa in density gradient centrifugation.
J Reprod. Fertil. 42, 587-591

ROSS, A., J. A. ROBINSON and H. J. EVANS (1975):
Failure to confirm separation of X- and Y-bearing human sperm using BSA gradients.
Nature 253, 354-355

ROSSELLI, M., R. K. DUBEY, B. IMTHURN, E. MACAS and P. J. KELLER (1995):
Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity.
Hum. Reprod. 10, 1786-1790

SAACKE, R. G., J. C. DALTON, S. NADIR, R. L. NEBEL and J. H. BAME (2000):
Relationship of seminal traits and insemination time to fertilization rate and embryo quality.
Anim Reprod. Sci. 60-61, 663-677

SARTORI, R., R. SARTOR-BERGFELT, S. A. MERTENS, J. N. GUENTHER, J. J. PARRISH and M. C. WILTBANK (2002):
Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter.
J. Dairy Sci. 85, 2803-2812

SCHENK, J. L., T. K. SUH, D. G. CRAN and G. E. SEIDEL, JR. (1999):
Cryopreservation of flow-sorted bovine spermatozoa.
Theriogenology 52, 1375-1391 article

SCHILLING, E., and D. THORMAEHLEN (1977):
Enrichment of human X- and Y-chromosome bearing spermatozoa by density gradient centrifugation.
Andrologia 9, 106-110

SCHOFF, P. K., and H. A. LARDY (1987):
Effects of fluoride and caffeine on the metabolism and motility of ejaculated bovine spermatozoa.
Biol. Reprod. 37, 1037-1046

SEIDEL, G. E., JR. (2003):
Economics of selecting for sex: the most important genetic trait.
Theriogenology 59, 585-598

SEIDEL, G. E., JR., C. H. ALLEN, L. A. JOHNSON, HOLLAND M.D., BRINK Z., WELCH G.R., GRAHAM J.K. and CATTELL M.B. (1997):
Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa.
Theriogenology 48, 1255-1264

SEIDEL, G. E., D. G. CRAN, L. A. HERICKHOFF, J. L. SCHENK, S. P. DOYLE, AND R. D. GREEN (1999):
Insemination of heifers with sexed frozen or sexed liquid semen.
Theriogenology 51, 400

SEIDEL, G. E., JR., and D. L. GARNER (2002):
Current status of sexing mammalian spermatozoa.
Reproduction. 124, 733-743

SEIDEL, G. E., JR., L. A. JOHNSON, C. A. ALLEN, WELCH G.R., HOLLAND M.D., BRINK Z., AND CATTELL M.B. (1996):
Artificial insemination with X- and Y-bearing bovine sperm.
Theriogenology 45, 309

SEIDEL, G. E., JR., J. L. SCHENK, L. A. HERICKHOFF, S. P. DOYLE, Z. BRINK, R. D. GREEN and D. G. CRAN (1999):
Insemination of heifers with sexed sperm.
Theriogenology 52, 1407-1420

SENGER, P. L., W. C. BECKER, S. T. DAVIDGE, J. K. HILLERS and J. J. REEVES (1988):
Influence of cornual insemination on conception in dairy cattle.
J Anim Sci 66, 3010-3016

SENGER, P. L., J. K. HILLERS, J. R. MITCHELL, W. N. FLEMING and R. L. DARLINGTON (1984):
Effects of serum treated semen, bulls, and herdsmen-inseminators on conception to first service in large commercial dairy herds.
J. Dairy Sci. 67, 686-692

SEVINC, A. (1968):
Experiments on sex control by electrophoretic separation of spermatozoa in the rabbit.
J Reprod. Fertil. 16, 7-14

SHASTRY, P. R., and C. HEGLE and S. S. RAO (1977):
Use of Ficoll-sodium metrizoate density gradient to separate human X-and Y-bearing spermatozoa.
Nature 269, 58-60

SHEKARRIZ, M., D. M. DEWIRE, A. J. THOMAS, JR. and A. AGARWAL (1995):
A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species.
Eur. Urol. 28, 31-35

SHIRAI, M., S. MATSUDA and S. MITSUKAWA (1974):
Electrophoretic separation of X- and Y-chromosome-bearing sperm in human semen.
Tohoku J Exp. Med. 113, 273-281

SHISHITO, S., M. SHIRAI and S. MATSUDA (1974):
Galvanic separation of X- and Y-bearing human spermatozoa.
Andrologia 6, 17-24

SIKKA, S. C. (1996):
Oxidative stress and role of antioxidants in normal and abnormal sperm function.
Front Biosci. 1, 78-86

SILLS, E. S., I. KIRMAN, L. T. COLOMBERO, J. HARIPRASHAD, Z. ROSENWAKS and G. D. PALERMO (1998):
H-Y antigen expression patterns in human X- and Y-chromosome-bearing spermatozoa.
Am. J Reprod. Immunol. 40, 43-47

SIRIVAIDYAPONG, S., F. P. CHENG, A. MARKS, W. F. VOORHOUT, M. M. BEVERS and B. COLENBRANDER (2000):
Effect of sperm diluents on the acrosome reaction in canine sperm.
Theriogenology 53, 789-802

SÖNDERQUIST L., MADRID-BURY N. and RODRIGUEZ-MARTINEZ H. (1997):
Assessment of ram sperm membrane integrity following different thawing procedures.
Theriogenology 48, 1115-1125

SPRENGER, E., N. BOHM and W. SANDRITTER (1971):
Flow fluorescence cytophotometry for ultra-rapid DNA measurements on large cell populations.
Histochemie. 26, 238-257

STEVENSON, D., and A. R. JONES (1985):
Production of (S)-3-chlorolactaldehyde from (S)-alpha-chlorohydrin by boar spermatozoa and the inhibition of glyceraldehyde 3-phosphate dehydrogenase in vitro.
J Reprod. Fertil. 74, 157-165

STOVEL, R. T., R. G. SWEET and L. A. HERZENBERG (1978):
A means for orienting flat cells in flow systems.
Biophys. J 23, 1-5

SUH, T. K. and J. L. SCHENK (2003):
Pressure during flow sorting of bull sperm affects post-thaw motility characteristics.
Theriogenology 59, 516

TENHAGEN, B. A., R. SURHOLT, M. WITTKE, C. VOGEL, M. DRILLICH and W. HEUWIESER (2004):
Use of Ovsynch in dairy herds--differences between primiparous and multiparous cows.
Anim Reprod. Sci. 81, 1-11

TENHAGEN, B. A., M. WITTKE, M. DRILLICH and W. HEUWIESER (2003):
Timing of ovulation and conception rate in primiparous and multiparous cows after synchronization of ovulation with GnRH and PGF2alpha.
Reprod. Domest. Anim 38, 451-454

THUN, R., M. HURTADO and F. JANETT (2002):
Comparison of Biociphos-Plus and TRIS-egg yolk extender for cryopreservation of bull semen.
Theriogenology 57, 1087-1094

TUBMAN, L. M., Z. BRINK, T. K. SUH and G. E. SEIDEL, JR. (2004):
Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting.
J Anim Sci 82, 1029-1036

UWLAND, J., and C. M. WILLEMS (1975):
[Results of semen separation using a modified electromagnetochemical method].
Tijdschr. Diergeneeskd. 100, 911-914

VAZQUEZ, J. M., E. A. MARTINEZ, I. PARRILLA, M. A. GIL, X. LUCAS and J. ROCA (2002):
Motility characteristics and fertilizing capacity of boar spermatozoa stained with Hoechst 33342.
Reprod. Domest. Anim 37, 369-374

VAZQUEZ, J. M., E. A. MARTINEZ, I. PARRILLA, J. ROCA, M. A. GIL and J. L. VAZQUEZ (2003):
Birth of piglets after deep intrauterine insemination with flow cytometrically sorted boar spermatozoa.
Theriogenology 59, 1605-1614

VERBERCKMOES, S., S. A. VAN, J. DEWULF and K. A. DE (2005):
Comparison of three diluents for the storage of fresh bovine semen.
Theriogenology 63, 912-922

VIDAL, F., M. MORAGAS, V. CATALA, M. J. TORELLO, J. SANTALO, G. CALDERON, C. GIMENEZ, P. N. BARRI, J. EGOZCUE and A. VEIGA (1993):
Sephadex filtration and human serum albumin gradients do not select spermatozoa by sex chromosome: a fluorescent in-situ hybridization study.
Hum. Reprod. 8, 1740-1743

VIDAMENT, M., P. ECOT, P. NOUE, C. BOURGEOIS, M. MAGISTRINI and E. PALMER (2000):
Centrifugation and addition of glycerol at 22 degres C instead of 4 degrees C improve post-thaw motility and fertility of stallion spermatozoa.
Theriogenology 54, 907-919

VISHWANATH, R., C. P. PITT and SHANNON P. (1996):
Sperm numbers, semen age and fertility in fresh and frozen bovine semen.
Proc NZ Soc Anim Prod 56, 31-34

VOLKMAN, D. H., and D. VAN ZYL (1987):
Fertility of stallion semen frozen in 0,5-ml straws.
J ReprodFertil Suppl 35, 143-148

WANG, G. L., B. H. JIANG and G. L. SEMENZA (1995):
Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1.
Biochem. Biophys. Res. Commun. 216, 669-675

WANG, H. X., S. P. FLAHERTY, N. J. SWANN and C. D. MATTHEWS (1994b):
Assessment of the separation of X- and Y-bearing sperm on albumin gradients using double-label fluorescence in situ hybridization.
Fertil. Steril. 61, 720-726

WANG, H. X., S. P. FLAHERTY, N. J. SWANN and C. D. MATTHEWS (1994a):
Discontinuous Percoll gradients enrich X-bearing human spermatozoa: a study using double-label fluorescence in-situ hybridization.
Hum. Reprod. 9, 1265-1270

WAY, A. L., and G. J. KILLIAN (2002):
Capacitation and induction of the acrosome reaction in bull spermatozoa with norepinephrine.
J Androl 23, 352-357

WELCH, G. R., and L. A. JOHNSON (1999):
Sex preselection: laboratory validation of the sperm sex ratio of flow sorted X- and Y-sperm by sort reanalysis for DNA.
Theriogenology 52, 1343-1352

Welch, G. R., G. C. Waldbieser, R. J. Wall, and L. A. Johnson (1995):
Flow cytometric sperm sorting and PCR to confirm separation of X- and Y-chromosome bearing bovine sperm.
Animal Biotechnology 6, 131-139

WHITTINGTON, K., and W. C. FORD (1998):
The effect of incubation periods under 95% oxygen on the stimulated acrosome reaction and motility of human spermatozoa.
Mol. Hum. Reprod. 4, 1053-1057

WILLIAMS, B. L., F. C. GWAZDAUSKAS, W. D. WHITTIER, R. E. PEARSON and R. L. NEBEL (1988):
Impact of site of inseminate deposition and environmental factors that influence reproduction of dairy cattle.
J Dairy Sci 71, 2278-2283

WOLFE, C. A., P. S. JAMES, A. P. GUNNING, S. LADHA, Y. CHRISTOVA and R. JONES (2001):
Lipid dynamics in the plasma membrane of ram and bull spermatozoa after washing and exposure to macromolecules BSA and PVP.
Mol. Reprod. Dev. 59, 306-313

ZAVOS, P. M. (1985):
Sperm separation attempts via the use of albumin gradients in rabbits.
Theriogenology 23, 875-879

12 Appendix

12.1 Materials

L-α-Lysophosphatidylcholine (L 5004); Lectin, FITC labelled from Arachis hypogaea (peanut) (L7381); Propidium iodide (P4170), 6-Carboxyfluorescein diacetate (C5041); Bisbenzemide H 33342 (B2261), Pyruvic Acid (P-3662) and Catalase (C-1345) were purchased from Sigma-Aldrich (Taufkirchen, Germany) all the other chemicals, if not specially stated, were purchased from Carl Roth GmbH + Co (Karlsruhe, Germany).

12.2 Composition of solutions

Tris-sample fluid: 199.98mM Tris-hydroxymethyl-aminomethan, 64.72 mM Citric acid monohydrate, 95.5mM D-fructose and 50mg Gentamicin Sulfat diluted in bi-distillated water.

Tris-BSA-sample fluid: Tris-sample fluid was supplemented with 3 mg/mL bovine serum albumin fraction V.

Hoechst 33342 working solution: 8.9 mM of 2-(-4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5-bi-1H-benzimidazole bi-distillated water.

Tris-sheath fluid: 197.13 mM Tris-hydroxymethyl-aminomethan, 55.34 mM Citric acid monohydrate, 47.46 mM D-fructose, 0.058 mg Penecillin G and 0.050 mg Streptomycin sulfat diluted in 5 L bi-distillated water.

Sodium Citrate sheath fluid: 149 g sodium citrate, 500 000IU Penicillin G and 0.25 g Streptomycin sulphate diluted in 5 L bi-distillated water. pH was adjusted to 6.9 with concentrated HCl solution.

TEST-yolk extender: 188.73 mM N-Tris-hydroxymethyl-methyl-2-aminomethan, 84.78 mM Tris-hydroxymethyl-aminomethan, 11.10 mM Glucose, 0.01 g Gentamicin – Sulfate and 5.0 g egg-yolk in 200 mL bi-distillated water. After centrifugation at 850 x g for 10 minutes, the supernatant was carefully removed and if necessary adjust to pH 7.4 with solid Tris-hydroxymethyl-aminomethan. Extender was frozen and kept in the freezer at – 20°C.

Tris egg-yolk freezing extender I: mixture of 67.2 mL stock solution (297.59 mM Tris-hydroxymethyl-aminomethan, 96.32 mM Citric acid monohydrate, 82.59 mM D-

fructose, 0.606 g Penicillin and 1.480 g Streptomycin sulfat per 1 L bi-distillated water.), 20.0 mL egg-yolk and 12.8 mL bi-distilled water.

Tris egg-yolk freezing extender II: the same as extender I, except that 12.8 mL bi-distillated water was replaced with 12.8 mL of 87 % Glycerol.

Food dye solution: 25 mg food dye FD&C#40 in 1 mL bi-distillated water.

Hancock solution: 2.784 g Tri-Natriumcitrat-Dihydrat, 4 mL 37 % Formaldehyde solution and bi-distillated water to 100 mL.

12.3 Tables

Table 12-1: Percentage of specific morphological abnormalities in unsorted and sorted spermatozoa submitted to different post sorting protocols

(n= 12)	Group A (%)	Group B (%)	Group C (%)	Control (%)
AL	2.3 ± 1.6	1.8 ± 1.4	1.9 ± 1.5	1.3 ± 1.0
AD	22.6 ± 4.6[a]	24.2 ± 6.6[a]	20.2 ± 5.5[a]	12.4 ± 6.9[b]
Aa	7.7 ± 3.8[e]	6.0 ± 2.4	7.1 ± 2.4[e]	4.2 ± 2.2[f]
Head	1.1 ± 0.9	1.2 ± 1.2	1.3 ± 1.8	1.8 ± 1.5
Neck	0.3 ± 0.6[e]	0.8 ± 1.1	0.3 ± 0.5[e]	1.4 ± 1.1[f]
Mid peace	3.6 ± 2.9	2.2 ± 1.9	2.9 ± 4.0	3.3 ± 1.7
PD	1.8 ± 2.5	1.6 ± 1.8	1.3 ± 1.6	2.3 ± 2.2
Coiled tails	4.3 ± 2.9[a]	6.3 ± 4.1[a.c]	2.5 ± 2.0[d]	1.8 ± 1.7[b]

AL: lost acrosomes; AD: dissolving acrosomes; Aa: other acrosome abnormalities; PD: protoplasmatic droplets

Values with superscripts differed significantly a:b and b:c (P<0.001); e:f (P<0.05).

Table 12-2: Viability and acrosome integrity of unsorted and sorted spermatozoa before and after LPC addition as tested with PNA/PI staining

n= 12	Group A (%)	Group B (%)	Group C (%)	Control (%)
		Before addition of LPC		
PI (-) / PNA (-)	36.8 ± 5.8[a]	40.2 ± 7.1	46.0 ± 5.6[b.c]	39.3 ± 9.5[d]
PNA (+)	24.3 ± 7.1[c]	20.8 ± 4.0[c]	22.1 ± 6.4[c]	34.5 ± 9.6[d]
PI (+) / PNA (-)	39.0 ± 8.0[a.c]	37.7 ± 5.6[a]	33.2 ± 5.6[d.e]	26.2 ± 6.2[b.f]
		After addition of LPC		
PI (-) / PNA (-)	27.3 ± 5.6	30.3 ± 6. 0	32.9 ± 6.6	29.7 ± 7.0
PNA (+)	40.6 ± 8.0[d]	32.2 ± 4.7[c]	33.4 ± 4.3[c]	41.9 ± 8.7[d]
PI (+) / PNA (-)	31.9 ± 7.2	36.1 ± 6.3[c]	34.8 ± 5.9[c]	28.3 ± 5.2[d]

Values with superscripts differed significantly a:b (P<0.001); c:d and e:f (P<0.05).

13 List of tables

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