Experiments to improve the quality of sex-sorted fresh and frozen porcine spermatozoa

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Man muß viel gelernt haben, um über das, was man nicht weiß, fragen zu können.

Jean-Jacques Rousseau

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List of Abbreviations

µl	micro litre
µm	micrometer
AI	artificial insemination
AO	antioxidants
ATP	adenosine triphosphate
BSA	bovine serum albumin
BSST	Beltsville Sperm Sexing Technology
cAMP	cyclic adenosine mono-phosphate
°C	degrees Celsius
Ca	Calcium
cm	centimetre
DUI	deep intrauterine insemination
ET	embryo transfer
et al.	et alii
FISH	fluorescence in situ hybridisation
FITC-PNA	fluorescein isothiocyanate labelled peanut agglutinin from Arachis hypogaea
FSH	follicle stimulating hormone
F/T	frozen/thawed
g	gravity
g	gram
h	hour
hCG	human chorionic gonadotropin
ICSI	intracytoplasmatic sperm injection
IUI	intrauterine insemination
IVF	in vitro fertilisation
kHz	Kilohertz
LH	luteinising hormone
LN_2	liquid nitrogen
mg	milligram
min.	minute

mm	millimetre
mMol	milliMol
n.s.	not significant
p	significance level
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	pondus hydro genii
PI	propidium iodide
PMSG	pregnant mare's serum gonadotropins
ROS	reactive oxygen species
SD	standard deviation
TALP	Tyrode, Albumin, Lactate, Pyruvat
UTJ	utero-tubal junction
UV	ultraviolet
≤	less or equal as
$\bar{x}$	Mean value

1. Introduction

Cryo-preserved boar sperm has been available for commercial purposes since 1975 (Westendorf et al. 1975, Johnson 1985). However, even though it is used in some commercial production occasions (Hofmo and Grevle 2000), for example in genetic transfer projects, frozen boar semen cannot be used under production conditions as efficiently as liquid-preserved semen. Reasons are the high susceptibility of boar spermatozoa to damage during cryo-preservation and a complicated process of deep freezing. Even though new trends like microinjection of dry frozen or dry fixed spermatozoa or culture and transplantation of spermatogonial stem cells are objective of research programmes, the liquid and cryo-conservation of spermatozoa remain as only choice for storage and commercial use of porcine semen (Yoshida 2000).

Weitze (2000) estimated that annually worldwide 155 million sperm dosages are produced for artificial insemination. From these 99% are preserved in a liquid form (Wagner and Thibier 2000). Liquid preservation ensures a storage time of about 3-5 with a maximum of 7 days (Weitze 2000). For international sperm exchange, cryo-conservation is a prerequisite. During storage in liquid nitrogen cell metabolism is almost completely inhibited. The only strain to cryo-preserved cells is the terrestrial background radiation and the half-life period of surviving spermatozoa is estimated to about 2000-4000 years (Mazur 1985).

Cryo-preservation is a prerequisite if specially treated or selected spermatozoa need to be stored. This is mainly the case when the capabilities for their production is limited as for example for gender preselection employing flow cytometry.

The only proven method to affect the gender of offspring is the flow cytometrical separation of X- and Y-bearing spermatozoa (Johnson et al. 2005a). With the latest generation of flow cytometers about 15 million spermatozoa can be produced per hour and sex. For non-surgical insemination of sexed spermatozoa a sperm dosage of at least 50×10^6 is required (Rath et al. 2003a, Grossfeld et al. 2005). As the life span of sorted fresh spermatozoa is lower than for unsorted equivalents, insemination has to be timed precisely and spermatozoa have to be inseminated close to ovulation. To setup efficient AI-logistics, freezing of sex sorted spermatozoa would be very advantageous if the post thaw quality would be acceptable.

Therefore the ultimate goal of the present study was to adapted existing freezing methods for porcine spermatozoa to the conditions of flow cytometrically sex sorted spermatozoa.

2. Review of Literature

2.1 Spermatozoa

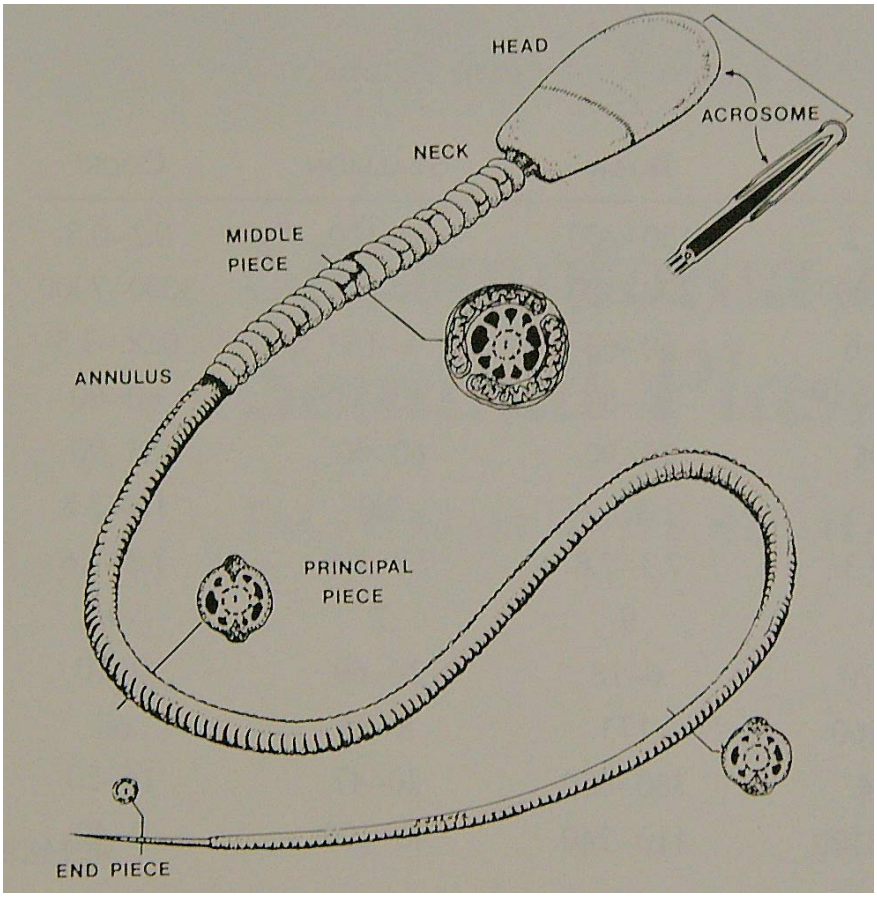
The male germ cell contains a haploid set of chromosomes. The cell fuses with the also haploid female germ cell, the oocyte, to build up the diploid zygote and to start the development of new life.
The sperm cell is able to fulfil a lot of functions in order to complete this task. The morphology of spermatozoa and their interaction with the varying environment in the female genital tract are of great significance (Fraser and Ahuja 1988).

2.1.1 Morphology and Motility

The sperm cell basically consists of head and tail. The latter is composed of the neck, mid, principal, and end piece.
The main part of the sperm head is the haploid nucleus, consisting of highly condensed chromatin (Wehner and Gehring 1990). Two third of the sperm head is covered with the acrosomal cap, which contains the necessary enzymes to penetrate the corona radiata and zona pellucida, i.e. hyaluronidase, acrosine and others (Leonhardt 1990). Acrosine has both proteolytic and binding characteristics (Töpfer-Petersen and Schill 1989).
The tail allows the sperm cell to actively penetrate the oocyte. The neck or connecting piece forms a basal plate that fits into depression in the posterior aspect of the nucleus. The basal plate of the neck continuous posterior throughout the most of the tail. The region to the tail between the neck and the annulus is the midpiece. The central core of the mid piece together with the entire length of the tail, comprises the axoneme. The axoneme itself is composed of nine pairs of microtubules that are located radial around two central filaments. In the middle this 9+2 arrangement of microtubules is surrounded by nine outer coarse of dense fibres that appear to be associated with the nine doublets of the axoneme. The axoneme and associated dense fibres of the mid piece are covered peripheral by numerous mitochondria. The

mitochondria are arranged in a helical pattern around the longitudinal fibres of the tail (Garner and Hafez 2000). The mitochondria provide energy by delivering ATP.
The principal piece, which continues posterior from the annulus extends to near the end of the tail, is composed centrally of the axoneme and its associated coarse fibres. A fibrous sheath provides stability for the contractile elements of the tail.
The end piece, which is posterior to the termination of the fibrous sheath, only contains the central axoneme covered by the plasma membrane. The axoneme is responsible for sperm motility. By cleaving ATP with dynein and kinesin molecules the outer pairs of microtubules of the 9+2 patterns generate the bending waves of the tail by a sliding movement between adjacent pairs (Lehninger et al. 1994).
The spermatozoa gain their capability of tail movement during their maturation in the epididymis. Mohri and Yanagimachi (1980) showed that hamster spermatozoa, recovered from the testicles and head of epididymis, were immotile. But spermatozoa recovered from the tail of the epididymis and ejaculated spermatozoa were motile.

Figure 1: Spermatozoa (Garner and Hafez 2000)



2.1.2 Biochemistry of membranes

Like all bio membranes the sperm membrane (plasma membrane) consists of proteins and lipids. The membranes grow and shrink with addition or withdrawal of lipids and proteins (Koolman and Röhm 1994). Gadella et al. (1999a) showed the highly dynamic structure of sperm membranes with their lipid composition. Fluidity, permeability, adhesivity and fusibility of the membranes can change due to external influences. The fluidity of membranes increases with temperature and with a growing amount of unsaturated fat acids (Koolman and Röhm 1994). The fluidity of the sperm head plasma membrane changes significantly during the cryo-preservation process (Canvin and Buhr 1989a, Buhr et al. 1989, Buhr and Pettitt 1996, Pettitt and Buhr 1998).

An intact sperm membrane is essential for full functionality of the sperm cell (Kumi-Diaka and Badtram 1994, Strom et al. 1997). The membrane structure of spermatozoa is highly correlated to extender components that support viability and motility of cryo-preserved spermatozoa (Pettitt and Buhr 1998). Alterations in membrane structure and function due to cryo-preservation are mainly responsible for reduced fertility of deep-frozen spermatozoa (Parks and Graham 1992b). Cryo-preservation of the plasma membrane leads to a redistribution of proteins in the sperm head membrane (De Leeuw et al. 1991), to alterations of the amount of or to the destruction of phospholipids and fatty acids (Parks and Graham 1992b) and partly also to the destruction of the plasma integrity (Almlid and Johnson 1988, Almlid et al. 1989a).

2.1.3 Capacitation

Chang (1951) and Austin (1951) noted that sperm must reside in the female reproductive tract before becoming capable of attaching to and penetrating the oocyte. The spermatozoa are stored in the epididymis until ejaculation. The protective measurements and stabilization of spermatozoa during their storage in the epididymis have a strong evolutionary correlation with necessity for capacitation in the female genital tract (Bedford 1994). To fertilize the oocyte the spermatozoa will

have to change from a stabilized status to an activated or capacitated status after ejaculation and deposition in the female (Töpfer-Petersen et al. 1996).

The capacitation process involves a series of endogen events like alteration of intracellular ion concentration, metabolism, redistribution of the sperm surface and a tyrosin-phosphorilation of sperm membrane proteins. These alterations are prerequisite for the zone pellucida induced acrosome reaction, the zone penetration and the fusion with the oocyte (Töpfer-Petersen et al. 1996, Kirchhoff 1995).

An important selection barrier for spermatozoa in the female genital tract is the utero-tubal-junction (UTJ). Only a few spermatozoa pass through the UTJ into the caudal isthmus (Hunter 1995). Obviously, the main events of capacitation take place in the lower segment of the isthmus (Hunter et al. 1987). Possibly, this is valid for all mammals, independent from the site of semen deposition (Yanagimachi 1994).

To initiate the capacitation as a process of progressive destabilisation, crucial for the later acrosome reaction, substances that are attached to or integrated in the sperm plasma membrane have to be removed or converted (Hunter and Greve 1998, Yanagimachi 1994). These substances are decapacitation factors (Fraser et al. 1990), Kaltrin, 15-, 16- and 23-kDa glykoproteins, all originating from the seminal plasma and 125-259-kDa proteins from the epididymis (Yanagimachi 1994). Approximately 50-60 million molecules bind through protein-protein- and protein-lipid-interaction, preferably to the acrosomal region of the sperm head. Protease inhibitors are classical decapaciation factors. In pigs their binding to the sperm surface is mediated by the interaction with sperm adhesines from seminal plasma (Töpfer-Petersen et al. 1996). During in-vivo and in-vitro capacitation more than 90% of the sperm adhesines together with the protease inhibitors are relieved again. The remaining part (approx. 6-8 million molecules) remains bound tightly to the surface and is involved in primary sperm binding to the zona pellucida (Töpfer-Petersen et al. 1995a). Subgroups of sperm adhesines are believed to have different functions. They serve as decapacitation factors as well as acrosome stabilizer. Parts of the sperm adhesines are removed from the sperm membranes during the migration of spermatozoa from the uterus to the lower isthmus either passively or by interaction of the sperm cells with the epithelia cells of the female genital (Calvete et al. 1997). In species with intrauterine semen deposition the spermatozoa appear to capacitate in the most, if not all in the lower isthmus (Yanagimachi 1994, Calvete et al. 1997). Spermatozoa are

capable to fully capacitate in the uterus without migrating to the oviduct (Brackett and Server 1970). They also can capacitate in the oviduct without any contact to the uterus (Bedford 1969). According to Bedford (1967) capacitation in rabbits is most effective when the spermatozoa have passed through uterus and oviduct.

Spermatozoa that underwent capacitation are membrane instable and short-lived (Bedford 1970). Therefore, capacitation has to occur in relation to ovulation. Hunter (1995) indicated that the full process of capacitation does not depend on the residence time of the spermatozoa in the lower isthmus, but is related to the time of ovulation. The time for boar sperm to fully capacitate takes about three hours (Hunter 1995).

According to Töpfer-Petersen et al. (1990, 1995b, 1996) capacitation leads to a total reorganisation of the sperm surface. During this process binding positions for the interaction with the oocyte and highly specialized micro-domains for fusion events of acrosome reaction are being developed. Furthermore, some second-messenger-systems and elements of signal-transduction are activated, which are prerequisite for the induction of the acrosome reaction by the zona pellucida. But the key event for the capacitation is the removal of cholesterol from the membranes (Davis 1981) and the influx of calcium ions (Harrison et al. 1993). Harrison (1997) showed that capacitation is a specific, initiable and controllable process, in which bicarbonate plays a key role. The influx of bicarbonate into the sperm cell leads to a stimulation of adenylat cyclase. Cyclic AMP production is initiated as a second-messenger, which activates protein kinase-A and simultaneously impedes the phosphotyrosin-phosphatase. Proteinkinase-A activates the protein-tyrosin-kinase, which finally causes capacitation (Visconti et al. 1998).

2.1.3.1 Influence of seminal plasma

During ejaculation spermatozoa are mixed with seminal plasma. Seminal plasma is the secretory product of the accessory glands in the male genital tract. Depending on species, the seminal plasma is build up by secretions of the ampoule, vesicular glands, prostate and bulbourethral glands. The secretions of the testicles and *Ducti deferentia* add only a minor part to the ejaculate (Töpfer-Petersen et al. 1998).

Seminal plasma is a complex mixture of different ions, low molecular organic substances, i.e. free amino acids, monosaccharides, lipids, polyamines,

prostaglandins, steroid hormones and proteins (Mann and Lutwack-Mann 1981). It is not surprising that interactions between sperm transport, capacitation and timing of ovulation, are affected in a synchronizing matter by seminal plasma (Weitze et al. 1990a, Waberski et al. 1996). The effects are mainly related to a special peptide fraction (1-10 kDa) induced by seminal plasma estrogens (Waberski et al. 1996). During oestrus it was possible to advance ovulation after early administration of seminal plasma. This effect was only significant in sows that showed a long "oestrus to ovulation" interval (Waberski et al. 1996). The advancement of ovulation is based on a shortage of the interval between LH-surge and ovulation. The time point of the LH-surge was not influenced by seminal plasma administration (Waberski et al. 1997). Interestingly, it was not possible to advance the time of ovulation in sows that had been treated with 750 I.E. hCG (Soede et al. (1998). The authors also noticed that the composition of seminal plasma varied among boars.

Seminal plasma has been described to have a positive effect on the accumulation of fertile spermatozoa in the oviduct, possibly due to an improved sperm transport. Viring and Einarsson (1980) described a relaxing effect of seminal plasma on the isthmus of the oviduct, which could be beneficial to the transport of spermatozoa to the site of fertilization. Waberski et al. (1996) and Soares (1995) determined a higher number of accessory sperm in the zona pellucida when semen samples contained seminal plasma. Even after AI with reduced sperm numbers (500 x 10^6 and 300 x 10^6) the authors did not notice any significant differences in fertilization rates when seminal plasma was present.

Claus et al. (1988) and Claus (1990) described the effect of seminal plasma on uterine contractions. Seminal plasma estrogens induced the release of prostaglandins from the endometrium and thereby strengthened uterine contractions. Additionally, seminal plasma estrogens seem to accelerate sperm transportation, which might be an important regulative part of fertilization.

Seminal plasma has direct influence on sperm motility by specific activating and impeding substances (Acott and Hoskins 1978, Iwamoto et al. 1992). Also capacitation is influenced considerably by decapacitation factors in the seminal plasma as described above. Chang (1957) noticed that sperm capacitation is delayed by seminal plasma. Further it was possible to decapacitate spermatozoa if seminal plasma was added to a solution containing already capacitated spermatozoa. There

are factors in seminal plasma, preventing capacitation and the subsequent fertilization unless removed (Dukelow et al. 1967). Also Calvete et al. (1997) described that removal of seminal plasma components has an important influence on capacitation.

Further, seminal plasma has an immuno-suppressive effect (Koch and Ellendorff 1985, Stanek et al. 1985) but partly also an immuno-stimulative effect in the female genital (Hadjisavas et al. 1994, Engelhardt et al. 1997). Immuno-suppressive components in seminal plasma with molecular weights of 100 and 110 kDa were detected in boar semen (Bouvet et al. 1987). According to Stanek et al. (1985) the immunosuppressive effect is mainly directed against B-lymphocytes. Spermatozoa have antigenic characteristics and function as chemo tactical mediators, leading to a migration of polymorphonuclear granulocytes (PMN) due to complement activation (Troedsson et al. 1995). The addition of 20 to 30ml of seminal plasma reduced the spermatozoa-induced migration of PMN in the uterus lumen (Rozeboom et al. 1999). However, Engelhardt et al. (1997) found a massive increase of neutrophile granulocytes and macrophages in the endometrium of gilts after administration of seminal plasma. Leshin et al. (1998) were able to show an immune stimulatory effect of seminal plasma proteins PSP-I and PSP-II on T- and B-lymphocytes in vitro. It is possible that certain seminal plasma proteins stimulate the proliferation of lymphocytes, which then interact with or synthesize immunosuppressive substances. Possibly, these substances protect spermatozoa, zygotes, and embryos against an immunological defence by the female genital tract (Leshin et al. 1998).

Taylor et al. (2007) showed that seminal plasma prevents spermatozoa from being bound to the uterine epithelial, as well as to neutrophilic granulocytes, suggesting an important protective role. They concluded that seminal plasma exerts a protective role that might be helpful in low dose insemination because it prevents the selection of viable spermatozoa in the uterus and therefore increases the number of spermatozoa reaching the UTV (Taylor et al. 2007).

Maxwell et al. (1996) and Maxwell and Johnson (1997) showed that viability as well as membrane integrity of ram-, bull- and boar-spermatozoa were reduced after flow cytometric sorting. The changes of spermatozoa during flow cytometry could partly be prevented by addition of 10% seminal plasma to the sorting medium (Maxwell et al. 1996).

Seminal plasma has an effect on the freezeability of boar spermatozoa. They are more prone to damage during cryo-preservation after contact to seminal vesicular secretions (Moore et al. (1977). Pena et al. (2003a, 2004b) and Selles et al. (2001) found differences in the post thaw sperm quality of different fractions of the ejaculate. In their study the sperm rich fraction tolerated handling and freezing better than the sperm poor fraction, indicating that seminal plasma has a negative influence on boar semen freezing.

2.1.4 Acrosome reaction

On successful binding of the spermatozoa to the oocyte the acrosome reaction is induced (Yanagimachi 1994). Only when spermatozoa are fully capacitated, they are capable to undergo acrosome reaction (Lee et al. 1987).

The induced acrosome reaction is distinguished from a spontaneous acrosome reaction. The spontaneous acrosome reaction takes place without influence of any external substances on the acrosome membrane, whereby the acrosome membrane lyses (Yanagimachi 1994). The induced acrosome reaction takes place on successful binding of the spermatozoon to the zona pellucida (Fazeli et al. 1997).

The acrosome reaction is a exocytotical process, whereby lytic enzymes are released. During the induced acrosome reaction, the outer acrosome membrane fuses at several locations with the zona pellucida. A cascade of different signal transductions is induced. The binding of the zona proteins to the receptors of the sperm membrane causes a depolarisation of the membrane and a stimulation of G-proteins. Calcium ion channels open and hydrogen ions are transported out of the cell. The intracellular calcium ion content increases. The pH-Value of the cytosol changes to alkaline. Caused by G-proteins, the concentration of the second messenger IP3 and diacylglycerol (DG) increases. DG is a regulating element in the phosphorylisation cascade and causes an increase of intracellular calcium. Furthermore DG activates cGMP-ase and cAMP-ase and causes an increase of cyclic nucleotides in the cytosol (Serrano et al. 2001).

The cytosol now contains a higher concentration of IP3. Calcium is released from intracellular storages and binds to calmodulin, whereby several proteins are phosphorylated. Finally, the increased intracellular calcium concentration causes the

acrosome reaction, with the release of the hydrolytic enzymes, stored in the acrosome. The acrosome reaction is essential for fertilization because the zona pellucida can only be penetrated by hyperactive spermatozoa, if a partial hydrolysis of the zona took place.

The binding and recognition of the two gametes is mediated by exposed carbohydrate-side-chains of the zona pellucida, which are being recognized by complementary receptors (zona-pellucida-binding proteins) of the spermatozoa (**primary binding**). After binding to the ZP, the acrosome reaction is induced. The released enzymes result in the lysis of the zona pellucida and enable the **secondary binding** of spermatozoa to the zona pellucida and further the penetration of the sperm cell into the oocyte. Acrosine is one of the enzymes that is activated and released by the acrosome reaction. It takes part in the penetration process together with other enzymes. Acrosin has, beside its proteolytic characteristics, also lectin-like affinities for the carbohydrates of the ZP. It is discussed that Acrosin is responsible for the secondary binding after the acrosome reaction took place, to assure that acrosome reacted spermatozoa do not swim away from the ZP (Töpfer-Petersen and Aurich 2000). After penetration of the ZP the fusion of the side by side laying plasma membranes of spermatozoa and oocyte take place.

2.2 Cryo-preservation methods for boar spermatozoa

During evolution spermatozoa have developed from a somatic cell to a very specialized gamete cell with the only task to guarantee the genetic maintenance of DNA information, including the transport through the female genital tract (Yoshida 2000). Preservation methods have to account for this and should not only allow the survival of spermatozoa for an unlimited time period but also must not reduce the fertilizing ability significantly.

2.2.1 History of Cryo-preservation

Already in 1776 Spallanzani was able to observe sperm immobilization by cooling/freezing when he exposed spermatozoa from humans, stallions and frogs to

snow (Spallanzani 1776). When he re-warmed them after 30 minutes they became motile again. A century later, Mantagazza (1866) observed that human spermatozoa survived when semen was frozen at -17°C. These two reports rank as the earliest in the recovery of mammalian cells after exposure to a temperature below their freezing point.

At least as important was the discovery of the cryoprotective potential of glycerol by Chris Polge (1949). He opened up an era of successful cryo-preservation methods, not only of gametes from various species, but also of other somatic cells and tissues. Fertility reports with deep frozen bull spermatozoa (Stewart 1951) led to intensive development of cryo-preservation methods that would be applicable for practical insemination purposes, including research efforts aiming at the development of a method for deep freezing of boar semen. Consequently, successful viability of frozen-thawed boar spermatozoa was reported (Polge 1956, Hoffmann 1959, Hess et al. 1960, Dukelow and Graham 1962, Bader 1964, King and Macpherson 1966, Kojima et al. 1967, Rohloff 1967, Bamba et al. 1968).

2.2.2 Development of cryo-preservation methods for boar semen

After the discovery of glycerol as cryo-protectant for spermatozoa by Polge et al. (1949) intensive research has been conducted to improve the survival of spermatozoa after thawing. The development of the cryo-preservation methods and composition of cooling, freezing and thawing solutions has been reviewed by Bwanga et al. (1991). The subsequent research efforts in boar semen cryo-preservation are summarized in Table 1.

Table 1: Recent reports about improving methods for cryo-preservation of boar semen

Area of Research	Material and Methods	Results	Reference
Container	Post-thaw motility and membrane integrity of spermatozoa frozen in maxi-straws or FlatPack-container with different freezing (20, 50, 80°C/min) and thawing (40s at 50° C or 27s at 70° C for Maxi-straws and 23s at 35° C, 13s at 50° C or 8s at 70°C for the FlatPacks) curves was evaluated	Freezing at a rate of -50°C resulted in slightly better sperm quality post thaw, semen frozen in FlatPack container showed better post thaw motility, due to more equal thawing of the sample	Eriksson and Rodriguez-Martinez 2000d
	Motility and membrane integrity of spermatozoa from nine boars frozen with a programmable freezing machine in plastic bags, 'cochettes', and in 'maxi-straws', in total doses of 5 x 10^9 spermatozoa/5 ml with glycerol (3%) used as cryo-protectant, were assessed after thawing	Cochettes sustained the overall procedure of freezing/thawing, with 30 min post-thaw sperm motility being significantly higher than for straws, 46.9 vs. 39.5%; percentages of FT-spermatozoa with intact membranes, detected with the supra-vital probes, were higher in maxi-straws than in cochettes, 46.8 vs. 43.0%; no significant differences were found in fertilizing capacity between spermatozoa frozen in maxi-straws and those frozen in cochettes	Eriksson and Rodriguez-Martinez 2000c
	Fertility of boar spermatozoa frozen in 0.5ml and 5ml straws was evaluated	Freezing in 5 ml straws did not have any detrimental effect on either penetration, monospermy, polyspermy, motility and NAR, in comparison with freezing in 0.5 ml straws	Cordova et al. 2001

Area of Research	Material and Methods	Results	Reference
	Frozen-thawed semen from 0.5 and 5 ml straws was compared to fresh semen and subjected to IVF after thawing	Semen frozen in 0.5ml straws showed higher rates for motility, NAR and lower rates for sperm penetration, monospermy, polyspermy, chromatin was significantly more unstable in sperm frozen in 0.5 ml straws	Cordova et al. 2002
	2 x 10^9 spermatozoa per ml were frozen either in 0.5ml medium straw, in 0.7ml FlatPack; 5 x 10^9 in a 5ml FlatPack served as control	Sperm motility did not differ between packages, viability was higher for 0.7ml FlatPack; spermatozoa could successfully be frozen in small volumes	Saravia et al. 2005
Freezing/Thawing curve	Three different freezing curves and three different container systems were compared	Best post-thaw sperm motility and percentage of NAR was obtained with semen frozen in 0.25ml Ministraws, 5ml Maxistraws and 5ml FEP-plastic bags, the best freezing curve was from +5 to -6°C at -3°C/min, hold 1 min at -6°C, at -20°C/min to -100°C	Bwanga et al. 1991a
	The effect of thawing velocities (from 10 to 1,800°C/min) in dependence from freezing rate (1-30°C/min) and glycerol level (2, 4, 6%) on sperm motility and NAR were tested	The best post-thaw motility (44-46%) was reached with 4-6% glycerol at a freezing rate of 30°C/min and a thawing rate of 1200°C/min; the percentage of NAR increased with the thawing rate, but was negatively affected by glycerol concentration at a freezing rate of 1°C/min	Fiser et al. 1993

Area of Research	Material and Methods	Results	Reference
	Three different freezing procedures were compared: 1) sperm rich fraction, cooled to 20°C and hold 1.5hrs, subsequent cooling to 15°C within 2.5hrs; 2) sperm rich fraction, cooling to +18 degrees C for 4 h and subsequent holding time at +18 degrees C for 16 h; 3) whole ejaculate (sperm rich fraction plus seminal plasma), cooling to +18 degrees C for 4 h and subsequent holding time at +18 degrees C for 16 h;	Sperm motility and NAR were significantly better with procedures 2 and 3 indicating a positive effect of extended holding time, exposure to seminal plasma had no significant effect	Kotzias-Bandeira et al. 1997
	The influence of prolonged storage of boar epididymes (0-3 days at 4°C) on post-thaw motility and IVF was evaluated	Data indicated that epididymal spermatozoa can be stored up to two days at 4°C prior to freezing before successful IVF	Kikuchi et al. 1998

Area of Research	Material and Methods	Results	Reference
	Semen was frozen in a split-sample design using 3 different holding times (3, 10 and 20 h) during cooling and three different types of freezing package: Maxi-straws, Medium-straws and FlatPacks; in vitro oocyte penetration ability of the spermatozoa was tested	Post-thaw sperm motility was significantly higher for 10 h and 20 h holding time compared with 3 h, and the percentage of motile spermatozoa decreased significantly with 20 h holding time as opposed to 3 h and 10 h; regarding the freezing packages, the FlatPacks and Maxi-straws yielded significantly more motile sperm than did the Medium-straws; motility was significantly higher for FlatPacks than for straws, in terms of both percentage motile spermatozoa, and sperm velocity and lateral head displacement; IVF did not show any significant differences among the holding time, although FlatPacks yielded a significantly higher penetration rate and more spermatozoa per penetrated oocyte ($p<0.05$) than did the straws	Eriksson et al. 2001
	Different freezing curves were used to freeze boar spermatozoa and evaluated for motility, viability, and acrosomal integrity in the membrane-intact population	-30 or -50 degrees C/min showed better results than -1 degrees C/min, with a slight advantage being evident for -30 degrees C/min	Kumar et al. 2003

Area of Research	Material and Methods	Results	Reference
	Two thawing procedures were analysed (37° C, 30 s; 50° C, 12 s) and thawed spermatozoa were subjected to IVF	No differences in sperm quality where found, with 50 degrees C, 12 s the IVF results showed a higher number of sperm per penetrated oocyte and a near 10 points higher rate of pronuclear formation	Selles et al. 2003
	New freezing technique was used; semen was diluted in L-EY, Glycerol diluents and cooled to -25°C at different rates in cryo-tubes in a liquid environment, samples were subsequently plunged into liquid nitrogen, thawing was done at room temperature for 45s and then for 5min at 37°C in a water bath, thawed semen was used for AI	Cooling of semen within 5 min to -10°C and then within 4min to -25°C showed best post thawing motility, motility was significantly better than control sperm frozen in straws, fertility trial proved fertility of spermatozoa frozen with new technique	Goolsby et al. 2004
	Three different centrifugation regimes (C1: 2400 x g for 3 minutes), (C2: 1600 x g for 5 minutes) (C3: 800 x g for 10 minutes) for sperm concentration at 15°C were compared	C1 and C2 showed significantly higher post-thaw sperm motility, viability, and percentage of non capacitated sperm; C1 had the highest oocyte penetrating ability and did not affect sperm yield	Carvajal et al. 2004
	Seeding of super-cooled sperm samples at -5 or -15°C was tested in a cryo-microscope	Percentage of membrane intact sperm was higher after seeding at -15°C, but percentage of NAR was higher in samples seeded at -5°C	Woelders et al. 2005

Area of Research	Material and Methods	Results	Reference
	Two different holding times (3 vs. 24 hrs) at 15°C were compared; two different extenders were also compared (BTS vs. Androhep Plus)	No differences in pregnancy rate at 23days was detected but embryo number was decreased after 24 hrs. holding time; increasing holding time did not affect post-thaw motility in BTS but decreased motility after increased holding in Androhep Plus	Guthrie and Welch 2005
Composition of media	Two concentrations of glycerol (2% vs. 6%) and three fractions of thawed spermatozoa (Percoll-Gradient centrifugation, Swim-up, supernatant fraction after centrifugation) where compared, f/t spermatozoa where subsequently used for IVF	Raising the glycerol concentration decreased the proportion of NAR, spermatozoa from supernatant fraction showed highest rate of penetration and polyspermy, penetration rate was similar to that with IVF of fresh semen, but polyspermy rate was lower, pronuclear formation was higher	Zheng et al. 1992
	The influence of 2-hydroxyproyl-beta-cyclodextrin (HBCD) exposure for 3hrs on post-thaw sperm prior to freezing in pellets was evaluated	HBCD significantly improved post-thaw motility and acrosome integrity was three-fold higher with 40mmol HBCD than in the control group possibly indicating a stimulating effect on the efflux of membrane cholesterol	Zeng and Terada 2000

Area of Research	Material and Methods	Results	Reference
	Effects of freezing diluents of differing levels of osmolality (225-580 mOsm/kg) on boar sperm cryo-survival were tested	Highest motility was found in the 420 mOsm/kg group, and progressive motility in the 420 to 580 mOsm/kg groups was higher than that in the hypo- (225 mOsm/kg) and iso-osmotic (290 mOsm/kg) groups; intact acrosomes of the spermatozoa frozen in the 510 and 580 mOsm/kg BF5 diluents were more numerous than in other groups; the 420 and 510 mOsm/kg groups yielded maximal values of post-thaw membrane integrity; study indicates that moderately hypertonic BF5 diluents are favourable for the cryo-preservation of boar spermatozoa in pellets	Zeng et al. 2001

Area of Research	Material and Methods	Results	Reference
	Spermatozoa were exposed to methyl-beta-cyclodextrin (MBCD) or a combination of MBCD and cholesterol-3-sulfate over a period of 3 hours while being cooled slowly from 25 degrees C to 5 degrees C, and were subsequently cryopreserved by the pellet method	In post-thaw spermatozoa the values of the intact acrosome, motility, progressive motility, progressive velocity, straightness, and linearity of the cell path increased greatly with the concentration of MBCD; lateral head displacement amplitude and the beat cross-frequency of post-thaw spermatozoa were not different among all treatments; addition of cholesterol-3-sulfate to the diluent containing MBCD abolished the protective effect against freeze-thaw injury that MBCD provides to spermatozoa; results indicate that cryosurvival of boar spermatozoa is enhanced by exposure to MBCD before freezing	Zeng and Terada 2001
	Effect of N-acetyl-D-glucosamine, glycerol concentration and equilibration time for the freezing of boar spermatozoa in 5 ml maxi-straws was evaluated	Best results were obtained with first diluent containing 11% lactose hydrate, 20% egg yolk and 0.05% N-acetyl-D-glucosamine in 100ml distilled water, and the second diluent containing 11% lactose hydrate, 20% egg yolk, 4% glycerol and 1% orvus es paste	Yi et al. 2002a

Area of Research	Material and Methods	Results	Reference
	Experiments were carried out to investigate the effect of N-acetyl-D-glucosamine, and to obtain additional information about the effect of Orvus Es Paste (OEP) and egg yolk concentration in the freezing of boar sperm in the maxi-straw	Diluent with 0.025 or 0.05% soluble N-acetyl-D-glucosamine in the first diluent, 0.5% final Orvus Es Paste concentration and 20% egg yolk concentration significantly enhanced NAR acrosomes and motility of boar sperm after freezing and thawing; no effects of N-acetyl-D-glucosamine among the diluents with or without N-acetyl-D-glucosamine at the second dilution	Yi et al. 2002b
	Effect of addition of either 500 or 1000 µg/ml hyaluronan prior to freezing on post-thaw sperm characteristics was evaluated	Hyaluronan supplementation improved sperm motility, decreased percentage of hyperactive spermatozoa and maintained membrane stability as assessed with merocyanine-540	Pena et al. 2004a

Area of Research	Material and Methods	Results	Reference
	Spermatozoa were frozen with three different types of avian EY (chicken, duck or quail)	Sperm frozen in medium containing chicken egg yolk displayed higher motility immediately after thawing, but there was no difference in the motility of sperm frozen with different types of egg yolk 3 or 6 h after thawing and maintenance at 37 degrees C; sperm frozen in media containing chicken or duck egg yolk had a higher proportion of intact acrosomes immediately after thawing than sperm frozen in medium containing quail egg yolk, but 6 h after thawing and maintenance at 37 degrees C the sperm that had been frozen in medium containing chicken egg yolk had a higher proportion of intact acrosomes than the sperm frozen in media containing duck or quail egg yolk	Bathgate et al. 2006
	Spermatozoa were frozen with the addition of low density lipoproteins (LDL)	LDL addition of 9% in the freezing media showed an improved protection of the DNA integrity as assessed by neutral comet assay	Jiang et al. 2006

Area of Research	Material and Methods	Results	Reference
	Freezing medium was supplemented with either platelet-activating factor (PAF) or a recombinant platelet-activating factor:acetylhydrolase (PAF:AH; Pafase) before or after freezing	Addition of PAF to the cryopreservation medium improved post-thaw motility immediately after thawing and after 3h incubation at 37 °C; Acrosome integrity was higher immediately after thawing and after 3 and 6h incubation at 37 °C when sperm were frozen in the presence of Pafase; Addition of PAF to sperm after thawing improved motility immediately post-thaw, compared with addition of Pafase or the control sperm with no supplementation of the medium; the beneficial effect was lost by 3h post-thaw	Bathgate et al. 2007
	Dialysis was used to remove low-molecular weight components (12-14 kDa) in boar semen,	Dialysis enhanced post-thaw sperm motility, plasma membrane integrity and mitochondrial function, but had no significant effect on recovery of spermatozoa with intact acrosomes. Furthermore, dialyzed spermatozoa exhibited higher ATP content compared with the control after freezing-thawing	Fraser et al. 2007

Area of Research	Material and Methods	Results	Reference
	Effect of lactose and glycerol concentration, as well as the equilibration time with glycerol was studied on motility, normal apical ridge, and chromatin state of boar spermatozoa after the freezing and thawing process	Results indicated that freezing spermatozoa in extenders with increasing concentrations of lactose adversely affected motility but provided a protective effect on acrosomes; increased lactose concentration induced higher chromatin condensation but maintained the same stability; increasing the glycerol concentration in the freezing extender from 4-6 to 8% led to higher motility and NAR as well as lower chromatin condensation and stability	Corcuera et al. 2007

Although several improvements have been made to freeze boar semen, no major breakthrough in cell survival could be realised leading to a broader utilization of frozen-thawed semen in porcine industry. Due to the fact that still many spermatozoa are lost during freezing and thawing, new research efforts aim to use frozen / thawed spermatozoa more effectively for insemination in order to ensure a sufficient sperm population to be built up as sperm reservoir at the UTJ. For fresh semen AI the introduction of non-surgical deep intrauterine insemination (DUI) made it possible to reduce the required sperm dose 20 to 80 times (Vazquez et al. 1999, Martinez et al. 2001). Roca et al. (2003) used the same insemination technique to reduce the sperm dose of frozen-thawed spermatozoa from 6 to 1 x 10^9 without compromising farrowing rate or litter size.

Several field trials have been conducted to approve the improvements of cryopreservation as shown on laboratory level. Table 2 summarizes the major publications since this has been last reviewed by Johnson (1985).

Summarizing these trials, it can be seen that nearly all authors still use lactose-egg yolk for preparation of the cooling and freezing media for boar sperm. Also the fertility of frozen boar semen is still reduced compared to AI with fresh semen, as expressed by lower pregnancy rates and number of piglets or embryo and this even though higher sperm doses than in AI with fresh semen were used. On the other hand, some huge field trials show, that frozen boar semen can be integrated into the animal industry and applied on farm level.

Table 2: Summary of fertility results with frozen/thawed boar semen, 1985-2007

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol		?	3×10^9	Cervical	172		66.8	?	It was not statistically proved that percentage of NAR had positive effect on fertility		Premzl 1985
Pellet (BF5)		In BTS at 42°C	6×10^9	Cervical	200	1	50% Farrowing Rate	7.4 P	A broad variety in fertility between boars and consignment was detected	Fertility results from six export consignments to four countries	Curnock and Reed 1985

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
Pellet	Glucose, EDTA, Saccharose	Dry at 42°C	?		5		40%	7.5 P	Six years of semen storage did not cause further substantial changes in the structural and functional characteristics of spermatozoa	Semen stored for 6 years	Kozumplik 1985
L-EY, OEP, Glycerol		50°C 50s	5 x 10^9	Cervical	132	1	58 % Farrowing rate	9.7 P	A higher but not	frozen in 5ml or	Almlid et al. 1987

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
					118	2	68% Farrowing rate	9.6P	significant farrowing rate was observed with double AI	2.5ml straws	
L-EY, OEP, Glycerol		50°C 40s	5×10^9	Cervical	26	1	73.1%	?	Conception rate was equal for 2 and 4% Glycerol but in vitro parameter were better with 4%	Frozen with 2% Glycerol in Maxi-Straws	Almlid et al. 1989b
			5×10^9	Cervical	26	1	80.8%	?		Frozen with 4% Glycerol in Maxi-Straws	

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol		Water bath, 52°C, 52s	6×10^9	Cervical	220	1	51.4% Farrowing Rate	8.8 P	Fertilizing results fort his method where similar to that of other reports	Frozen in 5ml Macro-Straws in batches of 150-300 straws in controlled-rate freezer	Hammit and Martin 1989
L-EY, OEP, Glycerol	Part of the semen was supplemented with seminal plasma after thawing	Water bath, 50°C, 45s for Macrotubes or 39°C, 20s for FlatPack	5×10^9	Cervical	110	1-2	73%	12.0 E	No differences in fertility were found due to packaging, No. of AI or thawing solution	Semen frozen either in 5ml Macrotubes or 2ml FlatPack	Stampa 1989

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol		Water bath, 52°C, 52s	6×10^9	Cervical	48 experimental farm	1	72.9%	9.5 E	Fertility of frozen semen on experimental farm was better than on private farm	Frozen in 5ml Macro-Straws either on experimental or private farm	Kuo and Chiang 1990
			6×10^9	Cervical	152 private Farm	1	50.7% Farrowing Rate	8.0 P			
L-EY, OEP, Glycerol (Westendorf)		40°C	3×10^9	Cervical	344	1	69.2% Farrowing Rate	10.1 P	Frozen semen was successfully used for in a field trial	Frozen in 0.5ml Straws	Premzl et al. 1990
Pellets according to Beltsville Method			$5\text{-}10 \times 10^9$	Cervical	51	2	28%	6.8P	PGF2alpha did not affect fertility but reduced litter size	5mg PGF2alpha to semen for first AI	Takes et al. 1990
					50	2	29.4%	8.5P		control	

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol (Westendorf)		thawed in media containing seminal plasma	?	Cervical	55	1	73%	10.8 E	Seminal plasma had no special effect on fertility		Weitze et al. 1990b
		no seminal plasma in thawing media	?		55	1	73%	13.2 E			
			?		55	1	73%	12.2 E	Double AI showed no beneficial effect		
			?		55	2	73%	11.9 E			
			?		58	1	69%	10.8 E	Differences in fertility were not significant	Frozen in 5 ml Maxi-Straws	
			?		52	1	77%	13.3 E		Frozen in 2 ml Flat-Straws	

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
Pellet-Method Skim Milk, Lactose, Glucose, Egg Yolk, Glycerol	Aminoacetic acid, Na-citrate	Dry 50-60°C for 30s	40-60 x 10^6	Cervical	68056	1	75.5%	9.5 P		Field results from 1981-1988 in PRC	Xu and Wu 1990
					1053	1	57.7%	9.2 P	Double AI improved fertility		
					548	2	77.0%	10.1 P			
			20-45 x 10^6		96	1	57.3%	9.9 P	Farrowing rate was better due to higher sperm dose		
			40-75 x 10^6		229	1	75.1%	10.2 P			
			40-60 x 10^6		38	1	76.3 %	11.0 P	No differences in fertility were found due to storage time	Semen stored for 2-3 month	
					37	1	78.4%	9.9 P		Semen stored for 3-8 months	

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
					21	1	81.0 %	9.2 P		Semen stored for 4 years	
L-EY, OEP, Glycerol		Water bath 50°C, 40s	5 x 10^9	Cervical	13	1	63%	?	More ova were fertilized after AI with semen frozen in plastic bags	Frozen in 2.5ml Maxi-straws	Bwanga et al. 1991b
L-EY, OEP, Glycerol		Water bath 50°C, 40s	5 x 10^9	Cervical	13	1	75%	?		frozen in 5ml plastic bags	
L-EY, OEP, Glycerol		Water bath 50°C, 40s	4 x 10^9	Cervical	82	1	61.2% fertilized ova		Flat PVC-tubes improved fertility of frozen semen	Frozen in round PVC-tubes (4ml)	Simmet 1993
L-EY, OEP, Glycerol		Water bath 39°C, 15s	4 x 10^9	Cervical	81	1	77.3% fertilized ova			Frozen in flat rectangular cross-section PVC-tubes (2ml)	

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol		?	5 x 10^9	Cervical	19	1	84.2%	91.8% viable E	AI after Ovulation decreased embryo quality	AI 6h before ovulation	Bertani et al. 1996
		?	5 x 10^9	Cervical	19	1	78.9%	84.6% viable E		AI 6h after ovulation	
Pellet-Method	5mg P4 added before freezing	?	6 x 10^9	Cervical	13	2	53.8%	5.71 E	Addition of P4 to semen had no effect		Castaneda Moreno et al. 1996
Pellet-Method	5mg P4 added after freezing	?	6 x 10^9	Cervical	13	2	46.1%	6.83 E			
Pellet-Method	No P4 added	?	6 x 10^9	Cervical	13	2	61.5%	8.25 E		Control	
L-EY, OEP, Glycerol		42°C, 45s	5 x 10^9	Cervical	69	2	88.4% Farrowing rate	9.9 P	PGF2a did not influence farrowing	Frozen in 5 ml Macrotube ;	Gil et al. 1996

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol			5×10^9	Cervical	59	2	86.4% Farrowing rate	11.2 P	rate but reduced litter size	Frozen in 5 ml Macrotube ; 5mg of PGF2alpha were added to the semen dose	
L-EY, OEP, Glycerol		50°C 25s	5×10^9	Cervical	16	2	64.3%	9.9 E	Cochette showed better post-thaw motility but fertility results were not different	Frozen in 5ml Maxi-Straws	Rodriguez-Martinez et al. 1996
L-EY, OEP, Glycerol		50°C 25s	5×10^9	Cervical	16	2	62.5%	9.4 E		Frozen in plastic Cochettes	
Kiev+EY (80:20)					21		57.1%	9.75 P			

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
Huelsenberg-Method					21		47.6%	9.8 P	no differences were found to new extender type	Control	Samoulidis et al. 1996
L-EY, OEP, 2% Glycerol		37°C, 3min	30 x 10^6	Fallopian tube	12	1	25% PR 8.3% FR	2 P	AI of epididymal sperm in fallopian tube was successful	Epididymal sperm/ Epididymis stored at 4° for 24h	Kikuchi et al. 1999
CIAP freezing method					199	1-2	79.9%	10.1 P	No differences in fertility were found compared to AI with fresh semen	Field results of a AI Station 1995	Thilmant 1999
					298	1-2	76.2%	9.4 P		Field results of a AI Station 1996	

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol			4×10^9	cervical	193	2-3	74%	9.97 P	Fertility trial to set up European pig cryobank was successful		Bussiere et al. 2000
L-EY, OEP, Glycerol		Water bath, 50°C; 13s	5×10^9	Cervical	227	2	75%	10.6 P	Semen frozen in FlatPacks can successfully be exported	Semen frozen in FlatPacks and exported to Taiwan or Ireland	Eriksson and Rodriguez-Martinez 2000a
L-EY, OEP, Glycerol		Water bath, 50°C; 20s	3×10^9	Cervical	20	1-2	60	9.6 E	No differences in fertility were found due to semen packaging	Frozen in 5ml Maxi-straws	Eriksson and Rodriguez-Martinez 2000b
		Water bath, 50°C; 40s	3×10^9	Cervical	20	1-2	65	9.7 E		Frozen in 5ml plastic film cochettes	

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol	Thawing diluent consisted of 20% EY, 80% Lactose, 400 I.U. Catalase/ml	Water bath, 50°C; 45s	?		47	1	86.4 Farrowing rate	10.1 P	EY and Catalase improved post-thaw motility and showed successful fertilization	Frozen in 5 ml Maxi-Straws	Kuo and Huang 2000
CIAP freezing method		Water bath, 55°C, 12s	3.8×10^9 in 5 medium straws		22		100%	11.4 P	Treatments showed no significant differences		Thilmant 2001
			3.8×10^9 in 10 mini straws		22		90.0%	11.8 P			
			1.9×10^9 in 5 mini straws		22		86.4%	11.1 P			

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, 3% Glycerol		Water bath, 50°C, 13s	5 x 10^9	Cervical	352	2	72%	10.7 P	Freezing in FlatPack may be a reliable alternative for the freezing/thawing of boar semen under commercial AI cond.	Frozen in 5ml FlatPacks	Eriksson et al. 2002
?									Freezing semen in magnetic field improved sperm quality	Frozen in Maxi-straws in magnetic-field	Masuda et al. 2002
L-EY, OEP, Glycerol, 10min in LN2 vapour		Water bath, 70°C, 6s		Cervical	12		25%	7,3 P	AI of frozen semen was successful	0.5 French straws	Nizanski and Bielas 2003

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol+BF5, Pellets			6×10^9	cervical		2	88.4%	Fertilized eggs	Transgenes of parents were successfully transmitted to piglets	Several litters born!	Sommer et al. 2002
L-EY, OEP, Glycerol			6×10^9	Cervical	30	1	83.3% Farrowing rate	8.1 P	MR-A THAW and DUI can improve field results due to better use of ejaculates	Sperm thawed in MR-A THAW solution	De Alba et al. 2003
			6×10^9	DUI	45	1	12.1 Farrowing rate	9.0 P			
			3×10^9	DUI	45		81.8 Farrowing rate	8.0 P			
L-EY, OEP, Glycerol		?	1×10^9	DUI	45	2	34.7 Farrowing rate	8.9 P	a dose of 250×10^6 sperm can be used with DUI without reduction of	Frozen in 0.5 French straws	Bathgate et al. 2003
		?	250×10^6	DUI	45	2	42.9 Farrowing rate	7.2 P			

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
		?	62.5 x 10^6	DUI	45	2	7.1 Farrowing rate	7.3 P	Fertility		
L-EY, OEP, Glycerol			6 x 10^9	Cervical	33	1	75.8%	9.6 P	Application of DUI provides acceptable fertility in weaned sows using a relatively low number of frozen-thawed spermatozoa	Timed OV	Roca et al. 2003
L-EY, OEP, Glycerol			1 x 10^9	DUI	49	1	77.6% Farrowing rate	9.31 P		Timed OV	
L-EY, OEP, Glycerol			1 x 10^9	DUI	40	2	70% Farrowing rate	9.25 P		Spontaneous OV	
L-EY, Glycerol; unique freezing technology in liquid to -25°C then plunged in LN2		Waterbath, 45s at room temperature, then 5min at 37°C	3 x 10^9	Cervical	4	2	50 %	7 P	New freezing technology could successfully be applied	Spontaneous OV; only one of two boars sired litter	Goolsby et al. 2004

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
L-EY, OEP, Glycerol		Water bath, 50°C; 40s	5 x 10^9	Cervical	9	1	88.9%	16.2 E	Increased holding time did not decrease Pregnancy-Rate but decreased the number of embryos	3h Holding in BTS	Guthrie and Welch 2005
					7	1	57.1%	13.1 E		3h holding in Androhep Plus	
					8	1	50.0%	10.9 E		24h holding in BTS	
					7	1	57.1%	6.7 E		24h holding in Androhep Plus	
L-EY, OEP, Glycerol		37°C; 20s	1 x 10^9	DUI	50	2	42 % FR	8.3 P	Increasing AI-Dose improved fertility results in pre- and peri-	AI pre-ovulatory	Bolarin et al. 2006
			1 x 10^9	DUI	94	2	83 % FR	9.4 P		AI peri-ovulatory	
			1 x 10^9	DUI	35	2	31.4 % FR	7.3 P		AI post-ovulatory	
			2 x 10^9	DUI	71	2	70 % FR	9.38 P			

Freezing procedure	Diluent/ additives	Thawing procedure	Insemination dose	Site of AI	No. insem.	AI per oestrous	% Pregnant	Embryos/ Piglets (E/P)	Conclusion	Comment	Reference
			1×10^9	DUI	74	2	57.8 % FR	9.1 P	ovulated sows		
L-EY, OEP, Glycerol at concentration of 2 x 10^9 per ml		35°C 20s	1×10^9	DUI	19	1	38.8%	5 E	Pregnancies can be established with semen frozen in high concentration; DUI-AI interval should be -8 to -4h to OV, MiniFlatpack showed advantages in populating sperm reservoir	AI 8h before ovulation	Wongtawan et al. 2006
			1×10^9	DUI	15	1	40%	5.5 E		AI 4h before ovulation	
			1×10^9	DUI	8	2	25%	7 E		AI 12 and 4h before ovulation	
			1×10^9	DUI	22	1-2	31.8%	6.2 E		Frozen in MiniFlatpack	
			1×10^9	DUI	20	1-2	40%	4.8 E		Frozen in 0.5 ml Straw	

2.3 Strains for boar spermatozoa during cryopreservation

Except in bulls and with surgical insemination (Polge et al. 1970) fertility results after AI with frozen semen have never reached to level for AI with fresh semen. According to Watson (2000) a combination of a lowered viability of the sperm and an impaired function of the surviving population are reasons for diminished fertility. The number of fertile spermatozoa, regarding to sperm motility and acrosome integrity, is about 50% lower in frozen/thawed as compared to fresh semen (Crabo 1991, Watson 2000). In addition there is an absolute loss of sperm cells during processing for cryo-preservation (Schrader 1976), and the number of available semen portions per ejaculate is further decreased. Cryo-preservation of boar semen is associated with different physical and chemical insults to spermatozoa, such as cold shock, osmotic stress, intracellular ice crystal formation and cryoprotectant intoxication during the freezing process (Mazur 1984).

At present, it is generally accepted that the consequences of sperm cryo-injury are impaired transport and poor survival in the female reproductive tract (Salamon and Maxwell 1995). When the interval between insemination of frozen/thawed spermatozoa and ovulation is reduced, fertilisation increases (Parrish and Foote 1986, Kemp and Soede 1997), supporting the hypothesis that sublethal cryo-damage is an effect of the freezing/thawing procedure.

2.3.1 Effect of cryopreservation on sperm cell organelles

Sperm Membrane

Sperm organelles are enveloped by a system of membranes. They are particularly vulnerable during cryopreservation, including the plasma membrane, the outer acrosomal membrane and mitochondrial membranes (Hammerstedt et al. 1990, Parks and Graham 1992a, Watson 1995).

Despite doubling the number of sperm (Pursel and Johnson 1975a) insemination with frozen/thawed boar semen reduces both the farrowing rate and the litter size (Johnson et al. 1981). However, the fertility of such semen is not correlated with sperm motility, and is only partly correlated with acrosomal integrity (Wilmut and

Polge 1977). Loss of function may therefore be due to changes in the plasma membrane of the sperm head (Bailey et al. 2000). Canvin and Buhr (1989b) applied fluorescence polarisation and found that cooling and reheating of porcine plasma membranes significantly alters the membrane molecular organization and thereby its functionality (Sklar et al. 1979). The authors further found that the fluidity and therefore molecular organization of plasma membranes from the of boar spermatozoa differ from the sperm head. Their results indicated a differential temperature sensitivity between head and the rest of the sperm body membranes (Canvin and Buhr 1989b). Other authors also found an increased fluidity in cold-shocked (Robertson et al. 1990) and frozen/thawed (Buhr et al. 1989) boar spermatozoa. The membrane changes induced by cold shock were not reversible upon rewarming. Cryo-microscopical examination of ram spermatozoa loaded with a marker for membrane integrity (FDA) revealed that exposure to low temperatures followed by warming differentially affected the plasma membranes over the principal-piece, midpiece, and head, with the head membrane inevitably damaged (Holt and North 1994). Further investigation showed a significant sperm membrane permeabilization after exposure to a high salt concentration followed by restoration of osmotic equilibrium as would be generated during a freeze-thaw cycle. Because of the temperature and osmotic effects, both freezing and thawing induced tremendous alterations in cell water volume, which usually causes considerable mechanical stress on the cell membranes (Hammerstedt et al. 1990, Noiles et al. 1993, Noiles et al. 1995).

At physiological temperatures, freeze-fracture electron micrographs of bull and boar head plasma membranes exhibit a random distribution of membrane proteins (De Leeuw et al. 1991). During cooling, phase transitions changed sperm membrane ultra structure such that particle-free regions in bovine and protein aggregation in porcine sperm replace the random distribution. Because boar spermatozoa are known to be sensitive to cooling, the differences between theses species may indicate variable degrees of damage (Bailey et al. 2000). These ultrastructural modifications were not fully reversible after rewarming.

Cryo-preservation does affect lipid composition and organisation of sperm plasma membranes in boars (Buhr et al. 1989). Maldjian et al. (2005) observed a significant reduction in cholesterol in spermatozoa after cryo-preservation, which was correlated

with an increased number of acrosome-reacted cells and changes in motility parameters.

Transbilayer-phospholipid asymmetry also was disrupted in ram sperm plasma membranes after cryo-preservation (Hinkovska-Galcheva et al. 1989, Muller et al. 1999). Ultra-structural damage to membranes due to cryo-preservation destabilized them, predisposing spermatozoa to gross morphological defects, such as missing and abnormal acrosomes (Robertson et al. 1990).

Phase transition and other ultrastructural modifications of the plasma membranes during cooling and re-warming may play a role in the poor fertility of cryo-preserved spermatozoa. It is likely that reorganization of sperm membrane lipids disturbs the lipid-lipid and lipid-protein interactions required for normal membrane function (Parks and Graham 1992a).

Plasma membrane disruption due to cooling or freezing favours the loss of cations and enzymes from spermatozoa (Harrison and White 1972), providing some explanation for early observations that cold shock irreversibly depresses sperm motility and metabolic activity (White 1993). Cold shock also destroys the selective permeability of sperm membranes to calcium, thus leading to excessive intracellular levels, which reduce motility and lead to necrosis (Simpson and White 1986, Robertson et al. 1990). Cryo-preservation, in the presence of a cryo-protectant, is considered to be a more moderate treatment than cold shock, since a subpopulation of spermatozoa survives and maintains fertilizing capacity, despite an overall reduction in the percentage of motile and viable cells. In contrast, the computer-assessed motility of bovine sperm immediately after thawing, is not correlated with fertility (Bailey and Buhr 1994). Additionally, despite preservation of adequate motility, cryo-preserved human spermatozoa exhibit significant membrane damage as indicated by subnormal hypo-osmotic swelling tests (Check and Check 1991).

Once spermatozoa approach the site of fertilization, they attach transiently to the oviductal epithelial cells, experience capacitation and hyperactivation, bind to the oocyte zona pellucida, undergo acrosome reaction, penetrate the zona, and then finally fuse with and penetrate the oolemma. Impaired sperm membrane function due to cryo-preservation inevitably affects each of these processes and diminishes the likelihood of a successful fertilisation. Poorly motile spermatozoa are less likely to

arrive at the site of fertilization in vivo or penetrate the oocyte. Moreover, the motile sperm population itself is adversely affected by cryo-preservation.

The structural reorganization of sperm head plasma membranes after freezing and thawing appears to disrupt the ability of spermatozoa to interact normally with cells of the female genital tract. In pigs, spermatozoa stored at 18°C penetrated fewer zona-free hamster eggs than did fresh spermatozoa, and penetration by cryo-preserved spermatozoa was markedly reduced (Clarke and Johnson 1987). Reduced sperm binding is likely a consequence of membrane injury, possibly by structural damage to the sperm receptors or by incomplete receptor aggregation.

After cryo-preservation, surviving boar, bull and human spermatozoa contained more intracellular calcium than before, reflecting impaired membrane selective permeability mechanisms (Robertson et al. 1990, Bailey and Buhr 1994, Mclaughlin and Ford 1994). The elevated intracellular calcium levels of cryo-preserved spermatozoa and their reduced capacity to maintain normal concentrations of this cation (Bailey and Buhr 1994, Mclaughlin and Ford 1994) may also partly explain the poorer fertility of sperm after thawing. Intracellular calcium levels increase during capacitation (Parrish et al. 1999), hyperactivation (Suarez et al. 1993), and the zona pellucida–induced acrosome reaction (Bailey and Storey 1994, Florman 1994). Such calcium regulatory mechanisms during capacitation (Parrish et al. 1999) and the acrosome reaction (Florman et al. 1998) are complex, involving intracellular stores and voltage-dependent calcium channels. Disruption of capacitation and/or the acrosome reaction would severely compromise the fertilizing potential of spermatozoa, which may account for observations that the in vivo fertility rates of cryo-preserved bovine semen are correlated with the ability of the spermatozoa to moderate internal calcium levels (Bailey and Storey 1994).

Watson (1995) suggested that cryopreservation-induced modifications to sperm membranes make them more reactive to their environment after thawing such that they are in a partially capacitated state. Perez et al. (1996) showed that cryo-preserved ram spermatozoa undergo capacitation more quickly than fresh controls as assessed by reactivity to calcium ionophore and the chlortetracycline (CTC) fluorescent assay. CTC fluorescence also revealed a greater proportion of capacitated spermatozoa due to cooling and immediately after thawing in bull (Cormier et al. 1997), ram (Gillan et al. 1997), and boar (Maxwell and Johnson 1997). Watson

(1995) suggested that cryo-preservation introduces a sublethal modification of the sperm membranes, which renders them more sensitive to the environment after thawing.

2.3.2 Significance of reactive oxygen species (ROS)

An increasing number of studies show that reactive oxygen species (ROS) play an important role in fertility. They are involved in physiological sperm function including hyperactivation, capacitation, acrosome reaction, and zona binding (Kodama et al. 1996, De Lamirande et al. 1997). Conversely, when the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS creates oxidative stress. ROS such as H_2O_2 are known to arrest motility and block oxidative metabolism in spermatozoa (Tosic 1947). In mice, ROS lower sperm penetration rates into the oocyte and block the sperm-egg fusion involving oxidation of sperm -SH groups (Mammoto et al. 1996). Sperm DNA damage caused by ROS also has been reported to have consequences for the post fertilization embryo development (Aitken et al. 1998).

Increased ROS production in both human sperm and seminal leukocytes during cooling to 4°C was reported by Wang et al. (1997). Mainly leukocytes produce large amounts of ROS if stimulated (Krausz et al. 1992). In somatic cells, ROS leakage from the mitochondrial respiratory chain has been evaluated at 2 to 5% (Boveris 1977), and this is probably a major source of ROS produced in sperm mitochondria. Aitken et al. (1997) hypothesized that a NADH/NADPH oxidase could exist on spermatozoa as it exists in many other somatic cell types. Specifically, the activation of an aromatic amino acid oxidase following the death of ram and bull spermatozoa has been identified as a major source of ROS production (Tosic 1947, Upreti et al. 1998). Release of such an oxidase from dead spermatozoa reduced the motility and viability of intact bull spermatozoa (Shannon and Curson 1972).

Unsaturated fatty acids, which predominate the composition of sperm membranes, are susceptible to peroxidation (Halliwell and Gutteridge 1984), and the consequences are numerous, ranging from membrane damage, inhibition of respiration to leakage of intracellular enzymes (White 1993). Especially boar

spermatozoa are sensitive to peroxidative damage due to the high content of unsaturated fatty acids in the phospholipids of the plasma membrane (Parks and Graham 1992a, Cerolini et al. 2000) and the relative low antioxidant capacity of boar seminal plasma (Brezezinska-Slebodzinska et al. 1995).

It has been demonstrated in human (Alvarez and Storey 1992), bull (O'flaherty et al. 1997) and mouse spermatozoa (Mazur et al. 2000) that cryopreservation is associated with oxidative stress. Moreover, freezing and thawing of bovine spermatozoa increased the generation of ROS (Chatterjee and Gagnon 2001b), causing DNA damage (Lopes et al. 1998), cytoskeleton alterations (Hinshaw et al. 1986), inhibition of the sperm–oocyte fusion (Aitken et al. 1989) and affecting the sperm axoneme, leading to loss of motility (De Lamirande and Gagnon 1992a).
Centrifugation which is part of the porcine semen freezing protocol, presents stress to spermatozoa and may cause an increase in lipid peroxidation (Shekarriz et al. 1995). This may be more obvious as spermatozoa are stored in liquid nitrogen that increases peroxidation of membrane lipids itself (Chatterjee and Gagnon 2001b).
Observations that addition of antioxidants to semen improves sperm quality provide indirect evidence for the damaging effects of ROS on sperm function. Studies to preserve unfrozen semen revealed that extenders supplemented with exogenous antioxidants such as Catalase, greatly aided to maintain sperm quality (Foote 1967). Inclusion of antioxidants such as alpha-tocopherol, catalase and ascorbate had a protective effect on metabolic activity and cellular viability of cryopreserved bovine (Beconi et al. 1991, Beconi et al. 1993, Bilodeau et al. 2002, Klinc 2005) and porcine spermatozoa (Pena et al. 2003c, Pena et al. 2004b, Roca et al. 2005b).

Table 3 gives an overview to antioxidants that were used to improve the quality of frozen/thawed semen.

Table 3: Antioxidants that have been used to improve the quality of frozen/thawed spermatozoa in different species

Antioxidant	Material and Methods	Result	Reference
ascorbic acid	Ascorbic acid was added to the freezing media of human sperm	Ascorbic acid did not show any effects on sperm motility and hyperosmotic swelling test	Askari et al. 1994b
	Ascorbic acid was added to the tris-based freezing-diluent for ram semen	Ascorbic acid had no positive effect and reduced motility in concentrations above 50 mMol	Sanchez-Partida et al. 1997
	Ascorbic acid was added to whole milk, glycerol extender of bull semen	Addition of antioxidants did not show any beneficial effects on sperm motility	Foote et al. 2002
	Ascorbic acid was added to the freezing media of equine spermatozoa	Ascorbic acid did not improve sperm motility, DNA fragmentation, acrosomal integrity, viability or MMP after thawing	Baumber et al. 2005
BHT butylated hydroxytoluene	BHT was added to Beltsville thawing solution in a concentration of 0.05 to 2 mMol BHT; Boar spermatozoa were subsequently cooled to 5°C	Boar spermatozoa acquired resistance to cold shock immediately after exposure to 0.2-2.0 mMol BHT as judged by motility (the proportion of motile spermatozoa) and acrosomal integrity.	Bamba and Cran 1992
	BHT was added to the freezing media for boar spermatozoa	Post thaw sperm survival was higher when sperm where frozen with 0.2, 0.4 and 0.8 mMol BHT, lipid oxidation was further reduced, embryo development after IVF could be improved with BHT	Roca et al. 2004
Catalase	Catalase was added to the freezing media of boar spermatozoa	Catalase alone or in combination with SOD improved post-thaw survival of boar sperm;	Roca et al. 2005b

Antioxidant	Material and Methods	Result	Reference
	Catalase was added to the freezing media of equine spermatozoa	Catalase did not improve sperm motility, DNA fragmentation, acrosomal integrity, viability or MMP after thawing	Baumber et al. 2005
	Catalase was added to cooling and freezing extender of bull spermatozoa after flowcytometrical sex sorting	Addition of antioxidants significantly improved post-thawing motility, acrosome integrity and were able to restore fertility of sex-sorted semen vs. control spermatozoa	Klinc 2005
Carnosine	Carnosine was added to TRIS-based freezing-diluent for ram semen	Carnosine had no positive effect and reduced motility in concentrations above 50 mMol	Sanchez-Partida et al. 1997
reduced glutathione (GSH)	GSH was added to whole milk, glycerol extender of bull semen	Sperm motility was improved by 6-11% after freezing with 0.5 mMol of GSH with and without SOD	Foote et al. 2002
	GSH was added to the freezing media of equine spermatozoa	GSH did not improve sperm motility, DNA fragmentation, acrosomal integrity, viability or MMP after thawing	Baumber et al. 2005
	GSH was added to the thawing extender of frozen-thawed boar sperm	GSH in the thawing extender decreased number of capacitated sperm, chromatin condensation and percentage of sperm with changes in sulfhodyl groups, IVF rates where improved	Gadea et al. 2005
Rebamipide	Rebamipide was added to the freezing medium for human spermatozoa	Addition of 100 and 300 µmol improved sperm motility after thawing	Park et al. 2003

Antioxidant	Material and Methods	Result	Reference
Superoxide dismutase (SOD)	Bull sperm were frozen in the presence of SOD	SOD decreased the number of capacitated sperm after thawing	O'flaherty et al. 1997
	SOD was added to whole milk, glycerol extender of bull semen	Addition of SOD did not show any beneficial effect on sperm motility	Foote et al. 2002
	SOD was added to the freezing media of boar spermatozoa	SOD alone or in combination with Catalase improved post-thaw survival of boar sperm	Roca et al. 2005b
	SOD was added to the freezing media of equine spermatozoa	SOD did not improve sperm motility, DNA fragmentation, acrosomal integrity, viability or MMP after thawing but SOD increase DNA fragmentation	Baumber et al. 2005
4-hydroxy-2, 2, 6, 6-tetramethylpeperidine (Tempol)	Tempol was added to whole milk, glycerol extender of bull semen	Addition of Tempol did not show any beneficial effect on sperm motility	Foote et al. 2002
2,2,6,6-tetramethylpeperidine-1-oxyl (Tempo)	Tempo was added to whole milk, glycerol extender of bull semen	Addition of Tempol did not show any beneficial effect on sperm motility	Foote et al. 2002
Vitamin E, TROLOX	Vitamin E was added to freeze/thawing media of human spermatozoa and motility changes after thawing were evaluated	Vitamin E had no effect on motility after freezing and thawing but showed a partial inhibitory effect on lipid peroxidation	Alvarez and Storey 1993
	Alpha-tocopherol was added to the freezing media of human sperm	Alpha-tocopherol showed little but significant positive effect on sperm motility	Askari et al. 1994b

Antioxidant	Material and Methods	Result	Reference
	Ram spermatozoa were frozen in the presence of vitamin E	Vitamin E improved cell membrane integrity and centrifugal counter-current distribution	Ollero et al. 1996
	Bull sperm were frozen in the presence of vitamin E alone or in combination with vitamin C	In both cases the number of capacitated spermatozoa were reduced after thawing, vitamin E further protected the plasma membrane integrity	O'flaherty et al. 1997
	Alpha-tocopherol was added to the freezing media of bull sperm	IVF results were reduced when semen was frozen with vitamin E	Dalvit et al. 1998
	Trolox was added to the egg-yolk based freezing medium for boar spermatozoa	200 µmol improved the post-thawing motility and mitochondrial activity significantly in both the sperm-rich and sperm-poor fraction of the boar ejaculate	Pena et al. 2003b, 2004b
	Alpha-tocopherol was added to the freezing media of equine spermatozoa	Alpha-tocopherol did not improve sperm motility, DNA fragmentation, acrosomal integrity, viability or MMP after thawing	Baumber et al. 2005
	Alpha-tocopherol was added to the freezing media of boar sperm	200 µg/ml to 1000µg/ml alpha-tocopherol improved sperm motility post thawing, 200 µg/ml alpha-tocopherol protected spermatozoa against lipid peroxidation	Breininger et al. 2005

Given their importance in fertility, a significant role of ROS in sperm cryoinjury is likely. In vitro capacitation and tyrosine phosphorylation of sperm proteins are correlated with superoxide anion generation in human spermatozoa (De Lamirande and Gagnon 1998). Conversely, both superoxide dismutase and catalase inhibited capacitation and tyrosine phosphorilation (Leclerc et al. 1997). Bailey et al. (2000) speculated that cryoinjury may be induced by ROS activity generated during sperm processing.

2.3.2.1 Nucleus; DNA-integrity

Fraser and Strzezek (2005) have shown that cryo-preservation of boar semen enhances oxidative stress, which has a detrimental effect on the sperm DNA integrity. In their study, applying the neutral comet assay, they found that DNA damage increased in cryo-preserved boar spermatozoa, regardless of the extender type and packaging material. Similar findings were reported for cryo-preserved human and stallion spermatozoa using the comet assay (Duty et al. 2002, Linfor and Meyers 2002). Fraser and Strzezek (2005) used the DNA-binding stain SYBR-14 and H33258 as well a single-cell electrophoresis assay. They proposed that also activation of endonucleases, released from deteriorated plasma membranes of frozen-thawed spermatozoa increase in post-thaw DNA damage. Mammalian spermatozoa contain a mechanism by which they can digest their own DNA when exposed to a stressful environment (Ward and Ward 2004). According to Sotolongo (2005), endonucleases present in sperm plasma membrane are activated by freezing and thawing. Extensive DNA damage, with loss of sperm motility and ability to fuse with the oocyte, has been observed during high oxidative stress in human and bovine spermatozoa (Aitken et al. 1998, Baumber et al. 2003). Although the involvement of ROS in cryo-induced DNA damage has not been fully elucidated, it has been assumed that under such conditions DNA is under constant attack from ROS inducing DNA breaks (Halliwell and Aruoma 1991).

As mammalian spermatozoa have been shown to lack a DNA repair system, it is envisaged to have a multi-level defence, which however do not completely protected from oxidative stress.

Addition of antioxidants to freezing and thawing extenders have been used to improve fertility (Pena et al. 2003b, Roca et al. 2005b).

2.3.3 Tests to evaluate sperm functionality

Table 4 provides an overview of in vitro tests to monitor deterioration of sperm membranes, organelles, DNA, and functionality. These tests can help to predict the fertility of a given sperm sample. In order predict the fertility accurately a combination of several tests is required (Gadea 2005, Woelders 1991, Waberski et al. 1999).

Table 4: Tests to monitor changes in sperm membranes, organelles, DNA, and functionality applicable to boar semen

	Probe or Test	Principle	Comments	References
Membrane and organelle integrity				
	Plasma membrane			
	Hoechst 33258, YoPro-1, propidium iodide, ethidium homodimer, ToPro-3, TOTO, Bromphenolblue	Membrane-impermeable fluorescent probe with affinity for DNA penetrate only penetrates membrane damaged spermatozoa (live stain)		Hong et al. 1988, Mclaughlin et al. 1992, Harrison et al. 1996, Pintado et al. 2000, Garner et al. 1986, Cheng et al. 1996, Haugland 2004; Schrader et al. 1986, Geisler 1990
	Fluorescein diacetat, carboxy (methyl) derivates (i.e. FDA, CFDA) SYTO-17, SYBR-14 carboxyseminaphthorhodal fluor-1 (SNARF-1)	Amphipatic probe pass intact sperm membranes and enter living sperm, entered probes are deacylated by intracellular esterases immediately leaving the probe membrane-impermeable, but probes can leave membrane damaged stains (dead stain) deacylation results in DNA-binding of this probe with fluorescent properties SNARF-1 is an intracellular pH-Indicator that diffuses into living cells; esterases present in live cells hydrolyze the acetoxymethyl ester leaving SNARF-1 trapped within the cell cytoplasm	Live and dead stains are often used in combination as counter stains are available as LIVE/DEAD Sperm Viability Kits	Donoghue et al. 1995, Garner et al. 1994, Garner and Johnson 1995 Thomas et al. 1997, Krienke 2003 Pena et al. 2005
	Hypo-osmotic swelling test (HOST)	Only membrane intact spermatozoa swell in hypo-osmotic conditions; the effect is more easily visible in the tail than the sperm head		Jeyendran et al. 1984, Jeyendran et al. 1992, Vazquez et al. 1997, Petrunkina et al. 2000

	Probe or Test	Principle	Comments	References
	Acrosome			
	Lectin conjugates of *Pisum sativum* (PSA), *Arachis hypogaea* (PNA), Triticum vulgaris (WGA), Concanavalia ensiformis (ConA), Ulex europaeus (UEA)	Lectins are conjugated with fluorescent groups and bind to specific carbohydrate moieties of glycoproteins exclusively localized in the acrosome; detection can be done in flow cytometry and life cell imaging microscopy with living and fixed and permeabilized spermatozoa; in living sperm the absence of fluorescence is indicative for an intact acrosome, in fixed and permeabilized sperm, full fluorescent sperm are considered intact	An array of fluorescent groups can be used in combination with the lectins, most common are FITC, TRITC and RPE, PNA labelling is specific for the outer acrosomal membrane, PSA labels acrosomal matrix glycoprotein	Holden et al. 1990, Szasz et al. 2000, Cheng et al. 1998, Flesch et al. 1998, Töpfer-Petersen et al. 1984, Malmi et al. 1987, Gadella and Harrison 2000, 2002, Gillan et al. 2005, Jimenez et al. 2002, Sinowatz et al. 1989
	Lysotracker™ dyes	Fluorescent dyes can be used to specifically stain the intact acidified acrosome	A variety of Lysotracker dyes with different absorption and emission spectra are available	Thomas et al. 1997, 1998
	lectin *Trypsin inhibitor* from Soybean (SBTI)	SBTI is specific for proacrosin and if conjugated to a fluorochrome, allows the recognition of acrosome damaged spermatozoa		Bussalleu et al. 2005
	Monoclonal antibodies (i.e. Mab ACR.2, mAk 18,6)	Monoclonal antibodies specific for either intra- or extra-acrosomal proteins are fluorescence-labelled can discriminate acrosome reacted spermatozoa		Peknicova and Moos 1990, Töpfer-Petersen et al. 1986, Bathla and Sidhu 1998, Töpfer-Petersen et al. 1988, Töpfer-Petersen et al. 1985, Zhang et al. 1990, Ivanova and Mollova 1993

	Probe or Test	Principle	Comments	References
	Morphological assessment	Determination of the percentage of spermatozoa with morphological abnormalities especially concerning the acrosome	A variety of dyes are available for acrosome staining and recognition of damages (i.e. Eosin, Farelly)	Krause 1966, Wells and Awa 1970, Würgau 1986, Lichtenstern 1995
	Mitochondria			
	Rhodamine 123, Mitotracker™	Only fluoresces in intact mitochondria when a proton gradient over the inner mitochondrial membrane is build up as a sign of an active aerobic ATP production, in unstained sperm the aerobic ATP production has stopped	Rhodamine 123 fluoresces red, Mitotracker TM are available with different absorption and emission spectra	Garner et al. 1997
	Mitotracker Orange CM-H2TMROS and Mitotracker X-Rosamine CM-H2XROS	Dyes become fluorescent in sperms with functional mitochondria undertaking oxidative respiration		Gadella and Harrison 2002, De Vries et al. 2003
	JC-1	Dye changes fluorescence properties due to changes in the potential of the inner mitochondrial membrane; JC-1 switches orange fluorescence in the aerobic functional mid-piece towards green in depolarized inner mitochondrial membranes		Garner and Thomas 1999
	Computer assisted sperm analysis (CASA)	Sperm motility characteristics (i.e. progressive motility, path velocity, curvilinear velocity) increase, when mitochondrial membrane potential increases	Alternative and indirect prove of mitochondrial function	Kasai et al. 2002, Pena et al. 2003b

	Probe or Test	Principle	Comments	References
	Lipid organisation			
	Chlortetracycline (CTC)	Fluorescent probe binds to sperm plasma membrane in a Ca2+/Mg2+ dependent manner; CTC stains viable sperm cells with the surface membrane containing Ca2+ above a certain threshold, the staining is scored in different pattern that allows conclusions about capacitation and acrosomal status	Probe cannot distinguish between calcium dependent and independent ways of capacitation, frozen-thawed sperm appear capacitated	Chandler and Williams 1978b, 1978a, Ward and Storey 1984, Saling and Storey 1979, Kaneto et al. 2002, Green and Watson 2001, Gadella and Harrison 2002
	Fluo-3-acetomethoxy(AM) ester, fura red-AM, quin-2 AM, indo-1 AM	Fluorescent probe can penetrate aqueous membrane compartments and change spectra when bound to calcium; probe delivers information about intracellular free calcium believed to be associated with capacitation		Green and Watson 2001
	Merocyanine 540 (M540)	Decreased packaging order of phospholipids in outer leaflet of the plasma membrane lipid bilayer, occurring during capacitation, allows probe to migrate into the hydrophobic core of the membrane and stain sperm cell;	M540 detects changes in sperm membrane organisations that happen earlier than detectable via CTC, M540 is also Ca2+ independent	Williamson et al. 1983, Langner and Hui 1993, Ashworth et al. 1995
	C6NBD-phospholipids	Incorporating, labelled phospholipid analogue is used to assess degree of phospholipids asymmetry in the plasma membrane of the sperm cell		Gadella et al. 1999b

	Probe or Test	Principle	Comments	References
	Annexin-V	Fluorochrome conjugated, calcium-dependent phosphatidylserine (PS)-binding protein; allows recognition of cells with exposed PS as in capacitated sperm		Gadella et al. 1999b
	Ro-09-0198	Fluorochrome conjugated, calcium-dependent phosphatidylethanolamine (PE)-binding protein; allows recognition of cells with exposed PE as in capacitated sperm		De Vries et al. 2003
	Lipid peroxidation			
	$C_{11}BODIPY^{581/591}$	Fluorescent analogue reporter probe for unsaturated fatty acids, probe changes its fluorescent properties when peroxidized by ROS or RNS	Location of peroxidation can be measured applying confocal laser scanning microscopy	Brouwers et al. 2005, Drummen et al. 2004
	Thiobarbituric acid reactive substance (TBARS)	The concentration of malondialdehyde (MDA) is measured by a spectrophotometer; MDA is produced due to peroxidative breakdown of membrane phospholipids		Comaschi et al. 1989
DNA damage				
	DNA condensation			
	Transmission electron microscopy (TEM)	Allows assessment of condensation status of single sperm cells; condensed nuclei appear homogeneously black in contrast to non-condensed nuclei	Can only be used in late spermatids prior to spermiation	Dooher and Bennett 1973, Roosen-Runge 1962
	Single cell DNA gel electrophoresis assay (COMET)	Normally condensed sperm nuclei (minimal migration) and loosely packed DNA (tailing of DNA) can be distinguished after fluorescent labelling of sperm DNA; tailing of DNA was noticed for mice with low expression of protamine 2		Cho et al. 2003

	Probe or Test	Principle	Comments	References
	DNA breaks and nicks			
	Sperm chromatine structure assay (SCSA)	Fluorochrome dye acridine orange stains single and double stranded DNA differently		Boe-Hansen et al. 2005, Evenson and Jost 2000
	TUNNEL-assay	Single stranded DNA in sperm cells is labelled with fluorescent nucleotide analogues by terminal nucleotide transferase; stained sperm contain therefore single-stranded DNA		Sakkas et al. 2003, Parrilla et al. 2003
	8-Hydroxydeoxyguanosine (8-OHdG)	8-OHdG is considered to be a precise and sensitive biomarker of oxidative DNA damage, level of 8-OHdG in sperm DNA is determined by high-performance liquid chromatography		Shen et al. 1999
	Chromomycin A_3 (CMA_3)	Fluorochrome detects protamine deficiency in loosely packed chromatin, which is correlated to the extend of nicked DNA; used to follow the last compaction steps of DNA to protamines, fluorescence dye binds to de-protaminated DNA but fails to do this after protamination of DNA		Manicardi et al. 1995, Catt et al. 1997, Sakkas et al. 1995
Sperm function and fertility				
	Computer assisted sperm analysis (CASA)	Sperm motility and motility characteristics (i.e. progressive motility, path velocity, curvilinear velocity) allow conclusions on sperm fertilization capacity	Sperm motility is not necessarily correlated with fertility; immotile sperm might be fully functional and regain their motility in the female genital, whereas motile sperm might have a damaged acrosome and cannot fertilize an oocyte	Holt et al. 1997, Popwell and Flowers 2004, Amann and Katz 2004

	Probe or Test	Principle	Comments	References
	Oviductal explant assay	(Electron-)Microscopically evaluation of sperm binding to oviductal epithelium cells in vitro reveals information about the capability of spermatozoa to form a functional sperm reservoir in the oviduct	Cultured explants, vesicles, apical membranes or monolayer of oviductal epithelium may be used	Waberski et al. 2006, Petrunkina et al. 2001, Raychoudhury and Suarez 1991, Fazeli et al. 1999, Green et al. 2001, Dobrinski et al. 1997, Boilard et al. 2002
	Sperm penetration assay (SPA)	The percentage of spermatozoa that enter zona-free hamster oocytes shows if spermatozoa are capable to successfully interact and penetrate an oocyte	Neither morphology nor the ability of the sperm to undergo an acrosome reaction during in vitro incubation was correlated with fertility in this study	Berger and Parker 1989
	Sperm-zona binding and penetration of pig oocytes	The capability of boar spermatozoa to bind to fresh or cryopreserved intact pig oocytes is evaluated to assess sperm functionality	The capability of sperm for zona-binding and zona-penetration may distinguished by passaging oocytes repeatedly through a narrow-bore pipette tip to strip off sperm bound to the zona surface	Ivanova and Mollova 1993, Lynham and Harrison 1998
	Hemizona binding assay (HZA)	The capability of boar spermatozoa to bind to the bisected hemizona of pig oocytes is evaluated to assess sperm binding capacity		Burkman et al. 1988, Fazeli et al. 1995

	Probe or Test	Principle	Comments	References
	Homologous in vitro fertilization	The capability of spermatozoa to produce embryos after in-vitro fertilization of in-vitro or in-vivo matured oocytes is measured to assess sperm functionality; the formation of pronuclei gives further information of sperm functionality	Homologous IVF-test have shown high variation between boars and ejaculates and are therefore criticized to not correctly predict fertility (Waberski et al. 2005)	Xu et al. 1996, Xu 1998, Long et al. 1999, Selles 2003, Miller et al. 1998
		The capability of spermatozoa to penetrate zona-intact but immature oocytes at germinal vesicle stage (hIVP) is measured to assess sperm functionality		Martinez et al. 1993, Vazquez et al. 1998
	Accessory sperm count and embryo recovery after AI in-vivo	Evaluation of fertilization rates, the proportion of normal embryos and number of accessory sperm give information about the capability of spermatozoa for oocyte recognition, oocyte binding and penetration and embryo development	This test was also used with hetero-spermic doses to identify the most capable spermatozoa	Ardon et al. 2003, Stahlberg et al. 2000, Hammitt et al. 1989
	Pregnancy rate and live born piglets	The ultimate information about sperm fertility is being delivered by the percentage of pregnant sows or number of piglets by themselves		

2.4 Gender preselection in animal breeding

Gender preselection is one of the most sought reproductive technologies in the animal breeding industry. Already 2500 years ago Greek philosophers reported how the gender of progeny might be influenced (Betteridge 1984). The prediction or determination of gender can either be conducted post-conceptionally by examination of early embryo or fetal stages or pre-conceptionally in mammals by separation of X and Y chromosome bearing spermatozoa (X and Y sperm). “Embryo-Sexing” is already being applied in farm animals, especially in cattle. The sexing is performed either by applying Polymerase Chain Reaction (PCR) or by in-situ Hybridisation with Y-Chromosome specific DNA probes (Herr et al. 1990, Levinson et al. 1992). Although embryo-sexing is already routinely conducted, sperm-sexing has got more advantages. Micromanipulation and the withdrawal of embryo cells are unnecessary. Sexed spermatozoa can improve the efficiency of breeding programs in important species significantly. Especially with gender related animal products or products that can be produced in a better quality and higher amount by one gender, inseminations with sex selected spermatozoa would be useful. The number of animals required to produce a certain amount of animal products could be optimized and the environmental pollution could be lowered. Examples for this would be meat and milk products in cattle and swine. In swine the required gender could be prepared for mating on the different levels of the breeding program. For fatting, female piglets are superior to the males with lower meat quality. Females are also superior in feed conversion ratio, daily gain rate (Niemann et al. 2003). Further, castration would be unnecessary as demanded from some European countries, where the use of castration will be outlawed and hence alternatives for producing higher numbers of females are required (Johnson et al. 2005a).

In cattle, the number of test matings to determine the breeding value could significantly be lowered without any loss in statistical power. On dairy farms preferentially female calves could be produced and likewise male calves on beef farms.

2.4.1 Methods of gender sperm sorting

In the past many different methods to predetermine the gender of offspring by sorting spermatozoa have been evaluated. Table 5 provides an overview of the different parameters. However, only the Beltsville sperm sexing technology (BSST) has been shown to produce repeatable and reliable gender predetermined offspring.

Table 5: Overview on parameters used or predicted to separate X and Y chromosome bearing spermatozoa

Parameter	Difference	Author(s)
DNA	X Sperm > Y	Moruzzi 1979, Pinkel et al. 1982
Size	X Sperm > Y	Cui and Matthews 1993, Cui 1997
Motility	Y faster than X	Ericsson et al. 1973
F-Body	Heterochromatic part on Y	Barlow and Vosa 1970
H-Y Antigen	sperm surface	Hendriksen 1999
Protein	sperm surface	Hendriksen et al. 1996
Surface charge	X migrates to cathode	Kaneko et al. 1984

2.4.2 Beltsville Sperm Sexing Technology

The sorting of spermatozoa into separate populations of X- and Y-bearing sperm based on DNA requires a flow cytometer. Sperm DNA has to be labelled with the fluorescent dye Hoechst 33342 (2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2′,5′-bi-1H-binzimidazole • 3 HCL). Hoechst 33342 (H33342) is a live-cell stain that permeates the cell membrane and binds selectively to A–T base pairs along the minor groove of dsDNA. H33342 usually is excited with the 351 or 364 nm UV light produced by an argon-ion laser (Seidel and Garner 2002). The slightly higher DNA content in X-chromosome bearing sperm cells results in a stronger fluorescent-signal

compared with Y-chromosome bearing sperm. Sorting purity ranges normally between 90% to 98% (Johnson 2000) using high speed flow cytometers.

Nowadays spermatozoa of the most species can be sorted with high purity if the difference in DNA content is at least 3%, requiring a correct sperm alignment in front of the laser and uniform labelling. The efficiency of sex sorting can be improved by adding the food dye FD&C#40. This dye exclusively stains spermatozoa with compromised membrane integrity and reduces the fluorescent signal of Hoechst 33342. It is therefore possible to exclude such damaged spermatozoa during sorting (Johnson and Welch 1999).

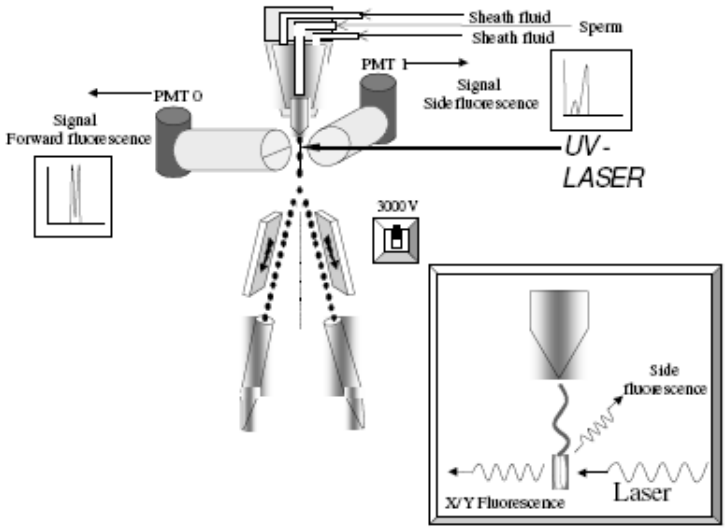
Johnson and Clarke (1988) showed the fertility of flowcytometric sorted sperm heads for the first time by injecting them into the cytoplasm of hamster oocytes. The first progeny out of sexed sperm after surgically inseminated of rabbits were derived by Johnson et al. (1989). The kittens did not show any morphological or genetic damages. From does inseminated with sorted X-bearing sperm, 94% of the offspring born were females. From does inseminated with sorted Y-bearing sperm from the same ejaculates, 81% of the offspring were males. At first a broader use of sexed spermatozoa was not possible due to low sorting capacity. Today modified high-speed flow cytometer are used for sperm sexing. The modifications consist of a forward fluorescence detector in place of the light scatter detector that is standard in orthogonal configured flow systems. This is necessary in order to collect the fluorescent light from both the edge of the sperm (90°) as well as from the flat side (0°) of the sperm. By means of electronic gating, one can collect edge fluorescence and eliminate much of the variability associated with differential fluorescence, thereby resolving the two populations. The second modification as originally designed, incorporates a bevelled sample injection needle to replace the usual cylindrical sample injection needle common to cell sorters. This bevel at the exit tip of the needle is designed to produce a flat ribbon type sample-sheath stream, which orientates the paddle shaped heads of the sperm cells to align them within the stream, so that as the spermatozoa pass the laser beam, a high proportion will be facing the beam in the proper plane. A significant improvement in the needle modification aspect was reported by Rens et al. (1998): the tip of the flow cell was remodelled to provide 2–3 times the orientation capability of the needle system. An elliptical shape

was modelled into the interior nozzle tip just above the exit orifice. The orienting tip of the nozzle brings the orienting action much closer to the laser beam detection system, thus increasing the orienting efficiency. Sperm sorters in use at the present time have incorporated the orienting tip design (Johnson and Welch 1999). XY. Inc. (XY® Sex Selection Technology and Services, Fort Collins, CO, USA) is furthermore selling sperm sorters with their own tip (Cyto-Nozzle®), which is an improved modification of the nozzle designed by Johnson.

The modified flow cytometer is essential for attaining separate populations of X- and Y-chromosome bearing living sperm (Johnson et al. 1989) on a repeatable basis. The modifications are also essential for reanalyzing sorted sperm for DNA to determine the proportions of X- or Y-sperm in a given sorted sample (Welch and Johnson 1999, Johnson et al. 1987). Differentiation of the amount of DNA present in the X- and Y-chromosome bearing sperm for sorting can be done on virtually all commercial cell sorters that have been manufactured in the past 30 years if they have been modified as described. The only aspect (in addition to improved optics) that is variable, is the rate of sperm being sorted and the subsequent sexed sperm throughput.

Figure 3 shows the principle of sex sorting mammalian spermatozoa in a flowcytometer.

Figure 2: Principle of a flowcytometer for sex sorting mammalian spermatozoa (modified Rath et al. 1996)



For the process of sex sorting spermatozoa, the semen is diluted and incubated with the fluorescent dye Hoechst 33342 for approximately one hour at 35°C. The incubation assists the penetration of the stain through the membrane. Uniform stain penetration is essential to minimize stain variation and helps to reduce the number of cells not being properly recognized by the system during the sperm separation. The result of proper processing is improved efficiency of sorting.

In the flow cytometer spermatozoa are carried with the sheath fluid to a piezo electric crystal undulating approximately 65.000 times/second, which breaks the stream into droplets at a particular point in time. The location of the last-attached droplet in the stream is highly controllable. Only droplets carrying a single cell are included into the sort decisions. Johnson et al. (2000) described PBS with 0,1% BSA as a carrier medium. Based on the difference of the dye related fluorescence signal sperm droplets are charged differently and are separated in the subsequent electrostatic field. The droplets are being collected in two collection tubes.

Only oriented sperm cells showing a defined DNA depended fluorescent signal are included in the sorting process (Rath et al. 1996).

2.4.3 Reanalysis

The validation of the sorted semen samples can be performed immediately after sorting either by reanalysis in the sorter (Welch and Johnson 1999), by fluorescence in situ hybridization (Kawarasaki et al. 1998) or by PCR (Welch et al. 1995). Sort reanalysis for DNA has the advantage over FISH and PCR, since both techniques may take up to 3 to 4 h, whereas reanalysis in the sorter requires less than 40 minutes. For a reanalysis in the flowcytometer 100.000 spermatozoa are taken. The cells are sonicated in order to remove the tails and Hoechst 33342 is added at tenfold concentration of the original concentration to maintain staining uniformity. The sperm cells are not sorted but analyzed at a very low speed to maximize orientation. DNA difference and histograms are analyzed by curve fitting to double Gaussian peaks (Johnson et al. 1987).

2.4.4 Influence of flow cytometry on spermatozoa

The fertility of sex sorted spermatozoa is lower compared to unsorted semen (Seidel et al. 1999, Buchanan et al. 2000). In trials of Doyle et al. (1999) and Seidel et al. (1999) the pregnancy rates of animals fertilized with sexed semen where 20% to 40% lower as the controls.

During sorting spermatozoa are exposed to different stress factors like high dilution, hydrodynamic pressure, UV light exposure and labelling with a critical dye (Johnson 2000). So far, the electric charge and the passage through the electro-static field seems to significantly affect motility (Garner 2006, Klinc and Rath 2007). However, besides reduced fertilizing capacity no changes have been identified so far, that would be critical for the use of sex sorted spermatozoa, especially no significant DNA modification could be identified (Parrilla et al. 2004).

It is accepted that sperm membranes change their patterns into the direction of capacitation. This however, is more a dilution effect that diminishes the presence of seminal plasma components known to be decapacitative. Small amounts of seminal plasma in the sheath fluid and in the collection medium allow an almost complete decapacitation. (Maxwell et al. 1996, Maxwell et al. 1998, Knöppel 2001). But the addition of 10% seminal plasma may have a negative effect on development rates

after IVF due to the decapacitating factors in the seminal plasma (Maxwell and Johnson 1997, Maxwell et al. 1998). However, boar sperm showed no reduced fertilization rates after IVF after the addition of 1% seminal plasma to the collection media (RATH, unpublished)

The addition of egg yolk to the collection media is also a prerequisite to maintain sperm viability after sorting (Johnson et al. 1989, Johnson 1991).

Johnson et al. (2005a) reported that an estimated many as 30.000 offspring, mostly cattle, have been produced in the past with sex sorted spermatozoa. Commercial application of sex sorted spermatozoa is practiced in cattle but not in the swine industry because of the large numbers of sperm that are required to achieve pregnancies.

The percentage of motile sperm cells is reduced by dilution with the sheet fluid (Ashworth et al. 1994). In addition to the osmotic effects, the high dilution of sperm cells obviously removes natural antioxidants and other useful substances descending from the seminal plasma, which are essential for the fertilizing capability of the spermatozoa (Ashworth et al. 1994).

The sorting process itself causes more alterations of the sperm membrane compared to spermatozoa that were only diluted, but not stained with Hoechst 33342 and sorted. The viability and membrane integrity of boar and ram sperm were reduced after sorting (Maxwell and Johnson 1997).

Preservation of maximal viability of sex sorted spermatozoa has not been fully achieved for the application for artificial insemination so far. The lowered viability is based on the progressive status of membrane maturation of the spermatozoa that were sorted. Maxwell et al. (1998) found that 55-65% of the sperm cells were already capacitated after flow sorting and 20-30% were already acrosome reacted. The precapacitation of sorted sperm cells was also described by Rath et al. (1997). In their trials treatments for capacitation of sperm cells for In-Vitro-Fertilisation were not necessary (Pursel and Johnson 1975b). Parilla et al. (2001) noticed a hypermotility of flowcytometrically sorted sperm cells also indicating a progressive capacitation status. Further stress for the semen cells arises during centrifugation in order to remove the sheath fluid (Maxwell et al. 1998).

Although there is no direct evidence that flow cytometrical sperm sorting causes lipid peroxidation, some reports raise some critical aspects. For flow cytometrical

sorting the spermatozoa have to be incubated with Hoechst 33342 for 1-1.5 hours, at a temperature of 34-37°C. At similar temperatures in mouse and rabbit spermatozoa the activation of high levels of energy for peroxidation processes has been detected (Alvarez and Storey 1985). Further the analysis of single spermatozoa during sorting demands a high dilution and consequently a concentration of cells by centrifugation after the sorting process. The natural defence against oxidation provided by seminal plasma is diminished by a high dilution during sorting with the sheath fluid. Addition of seminal plasma to samples during and after sorting has shown to have protective effects on the viability of spermatozoa (Maxwell et al. 1996). Centrifugation after sorting causes an increase in lipid peroxidation itself (Shekarriz et al. 1995). And finally, spermatozoa can be protected against lipid peroxidation during sorting. Antioxidant substances like Catalase have been used successfully for protection of bull spermatozoa during sorting and cryopreservation (Askari et al. 1994b, Beconi et al. 1993, Calamera et al. 2001, Bilodeau et al. 2002, Klinc 2005).

2.4.5 Cryopreservation of flow cytometrical sorted spermatozoa

Assuming a flow cytometer runs for 24 hours, 250 to 350 million of sexed spermatozoa for each sex with a purity of about 90% can be produced. The regular intracervical insemination in pigs requires at least 1 billion spermatozoa for each service. Due to the imprecise heat detection most animals have to be inseminated twice within 12 to 24 hours (Reed 1982, Hofmo 1991, Crabo and Dial 1992, Flowers and Esbenhade 1993, Steverink et al. 1999). Even if deep intrauterine insemination with low doses would be used to inseminate synchronized sows, a single sperm dose of 50 x 10^6 would be required. (Bathgate 2004, Grossfeld et al. 2005). This sperm dose has to be readily available at the farm at the right time for insemination. Other, more invasive technologies that only require a minimum number of spermatozoa for fertilization can be used as well: Johnson et al. (1991, Johnson et al. 2000) used fresh and frozen/thawed sex-sorted spermatozoa for surgical AI into the oviducts. Rath et al. (1997) successfully produced piglets with IVF/ET and surgical AI with only 400.000 sperm cells per ml of fertilizing media or AI respectively. Probst and Rath (2003) used ICSI to fertilize oocytes with sex sorted spermatozoa and achieved

thirteen piglets from four sows. However these techniques have the disadvantage that they require surgical insemination.

It would therefore be useful to be able to store sex sorted semen for an extended period and to be able to transport the semen easily. This could be done by the cryopreservation of sex sorted spermatozoa.

Up until now, there are only a few reports about successful deep freezing of sex sorted boar spermatozoa. Johnson et al. (2000) were able to establish pregnancies after laparoscopic insemination with sex sorted and frozen semen that resulted in the birth of two piglets. They estimated that up to 30% of the sperm cells were able to survive sorting and freezing according to the motility of the spermatozoa. Bathgate et al. (2005) used sex-sorted frozen/thawed semen for in-vitro fertilization. They were able to establish pregnancies but did not get any piglets. Deep intrauterine inseminations (Martinez et al. 2001) with sexed and frozen/thawed spermatozoa in another trial resulted in one pregnancy (Bathgate 2004). The cryopreservation of sorted spermatozoa of cattle is commercially used and has been successful in other species as well, as reviewed by Garner et al. (2006) and others (Maxwell et al. 2004, Seidel 2003, Klinc 2005).

2.5 Conclusions of the review of the current literature

The use of sex sorted boar semen is of great significance for the animal industry. Restricted amounts of flow cytometrically sorted spermatozoa are available. The available spermatozoa are damaged during the sorting process, which leads to a compromised fertility.

The improvement of cryo-preservation of boar spermatozoa is the centre of many research efforts. Improvements have been gained with the design of freezing and thawing protocols, the development of packaging units, the composition of semen diluents and more recently with the addition of antioxidants to these diluents.

The research of the current work aims to improve the cryo-preservation of boar spermatozoa in common and sex sorted boar spermatozoa in particular.

3. CHAPTER 1: Quality assessment of frozen/thawed boar semen against individual freezing curves in the presence of antioxidants

3.1 Introduction

Although cryo-preservation of boar semen for artificial insemination (AI) was developed some 35 years ago it still hampers to produce sufficient pregnancies with acceptable litter sizes. Even with excessive numbers of spermatozoa (5-6 x 10^9) thawed semen does not reach the fertility level of AI with fresh or cooled semen and represents the major limitation to its application in commercial AI programmes (Roca et al. 2006b). In an effort to improve the semen quality after freezing and thawing of boar semen two approaches were made. The first experiment evaluated the effect of three antioxidants (Pyruvate, Catalase and Mercaptoethanol) that principally act as ROS scavenger in cooling and freezing media (Klinc 2005) and in the second experiment three different freezing curves for individual boars were tested in the presence of these three antioxidants.

Cryo-preservation and the involved sperm processing have been described to support the creation of reactive oxygen species (ROS) (Alvarez and Storey 1985, Alvarez and Storey 1992). Porcine sperm membranes contain a relatively high proportion of polyunsaturated fatty acids, which decrease significantly during cryo-preservation, indicating lipid peroxidation (Cerolini et al. 2001). Lipid peroxidation has been correlated to exposure of spermatozoa to ROS and excessive ROS formation during cryopreservation has been associated with a decrease in the function of thawed spermatozoa (Chatterjee et al. 2001). Several antioxidants have been shown to improve the viability and functionality of cryopreserved boar spermatozoa. These include BHT, catalase (Roca et al. 2004), superoxide dismutase (Roca et al. 2005b, Bathgate 2004), Trolox (Pena et al. 2003b, 2004b) and other antioxidants. A combination of antioxidants has successfully been integrated in cryo-preservation of bull spermatozoa (Klinc 2005), where it improved sperm motility, viability and membrane integrity (Klinc et al. 2007, Klinc and Rath 2007). The goal of this

experiment was therefore to test if, Catalase and Mercaptoethanol in the presence of Pyruvate allow to improve sperm quality of cryo-preserved boar spermatozoa. Sperm motility and its resistance against incubation at body temperature, sperm morphology and membrane integrity were criteria to assess possible effects.

Roca et al. (2006b) reported that optimal sperm cryo-preservation does not only depend on the cryo-preservation methodologies, but also on the individual variation among boars to sustain cryo-injury. Further, Medrano et al. (2002) described the influence of different cooling curves on the survival of spermatozoa from individual boars. The aim of the second study was therefore to test whether an optimal freezing curve can be set for individual boars when ROS scavengers are present during the freezing process.

3.2 Material and methods

3.2.1 Freezing of boar semen in the presence of ROS scavengers

3.2.1.1 Processing of semen

Semen was collected twice weekly from four boars with proven fertility. Animals were housed at the Sydney University Research Farm at Camden, Australia, using the gloved-hand-method. About 50ml of the sperm-rich fraction were collected in a thermos receptacle in collection bags (Minitube of Australia, Sebastopol, Australia) and the Bulbourethral secretion was separated by a filter included in the collection bag. Directly after collection the semen was diluted with pre-warmed (38°C) Androhep^{TM} semen extender (Minitube of Australia, Sebastopol, Australia) (1:1, v/v). Motility of the samples was checked on a warming stage under a phase contrast microscope (Olympus CX 20, Olympus) at 100 x magnification. Semen was filled in pre-warmed 50ml plastic tubes (Greiner Bio-One, Tokyo) wrapped in a paper towel and transported to the laboratory in a cooling box within one hour at 15°C. Sperm concentration was determined in an improved Neubauer haemocytometer. An aliquot of 50µl was fixed in 500µl Glutaraldehyde-solution (3% in PBS, Sigma, St.Louis, MO, USA) to examine the morphology under a phase contrast microscope (Olympus CX 41, Olympus) at 1000x magnification under oil emersion. At least 200

spermatozoa were examined. The semen samples were then diluted with Androhep™ to a concentration of 4 x10^7 spermatozoa per ml in 15ml centrifugation tubes (Greiner Bio-One, Tokyo, Japan) .

A computer assisted sperm analysis (CASA) was made in a Hamilton-Thorne Sperm analyser (Hamilton-Thorne, Biosciences, Beverly, MA, USA) using 15µl of the diluted semen. Semen was then centrifuged for 10min at 800 x g in a cooling centrifuge (IEC Centra-7R, International Equipment Company, MN, USA) and the remaining sperm pellet was re-suspended with one of the extenders to be tested. Cryo-preservation was performed according to the Westendorf-protocol (Westendorf et al. 1975).

3.2.1.2 Experimental design

After centrifugation, split samples of semen were submitted to either one of three different treatments. Pellets were re-suspended with cooling extender (20% egg yolk, 80% Lactose-solution 11%, v/v) and extended to a concentration of 4x10^7 spermatozoa/ml.

In treatment group A the cooling extender contained 12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol per ml (all from Sigma, St. Louis, MO, USA). In treatment group B the cooling extender contained 200 µmol of the Vitamin-E-Analogue Trolox (6-hydroxy – 2,5,7,8-tetramethylchroman -2-carboxylic acid, Trolox™, Sigma, St.Louis, MO, USA). Group C served as control and contained neither antioxidants nor Pyruvate.

After re-suspension and thorough mixing, semen was cooled to 5°C within 2 hours. Thereafter in a cooling room, semen was slowly mixed with freezing extender, consisting of 92.5ml of cooling extender, 6ml glycerol and 1.5ml Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA). Two parts of semen to one part of freezing extender where mixed, giving a final glycerol concentration of 2%. The final sperm concentration was 2.67 x10^7 spermatozoa/ml. The samples were then loaded in 0.5ml straws (IMV, L'Aigle, France) and transferred to the chamber of a programmable freezer (Planer Products Kryo 10, Series III; Planer Products Ltd., Sunbury, Middlesex, UK). The cooling rate was as follows: -3°C/min from +5 to -6°C, one minute holding time at -6°C and thereafter -30°C/min to -140°C. The frozen straws were then plunged in liquid nitrogen (LN_2, -196°C) and stored until

thawing. For post-thawing semen evaluation the samples were removed from the LN_2 and thawed in a water bath at 38°C for 30s.

3.2.1.3 Analysis of thawed semen

The thawed straws were emptied in 5ml thawing tubes (Greiner Bio-One, Tokyo, Japan) kept in a water bath at 38°C. An aliquot of 120µl was put in a second tube containing 240µl Androhep™ and kept at 38°C until evaluation. The motility of the frozen/thawed semen was analyzed 10min, 1h, 2h, 3h and 6h after thawing under a phase-contrast microscope (Olympus CX 41, Olympus, Japan) equipped with a heating plate (Minitüb, Tiefenbach, Germany) at 38°C. Two drops and at least three fields per drop were analysed in each sample at 100x magnification.

For analysis of sperm morphology an aliquot of 120µl thawed semen was mixed with 200µl Glutaraldehyde-solution. Morphological abnormalities and acrosome integrity were analysed under a phase-contrast microscope (Olympus CX 41, Olympus, Japan) at 1000x magnification.

Membrane integrity and their functional status (acrosome reaction) were analysed flow cytometrically applying a triple staining method with SYTO-17/FITC-PNA/PI (Garner et al. 1999, Krienke 2003). After thawing, samples were diluted (1:2, v/v) right before staining to a final concentration of $5x10^6$ spermatozoa/ml with Androhep™.

Thereafter, 500µl of diluted semen were stained with 2µl SYTO-17™ (0,5 mM; from Molecular Probes), 4µl PI (2,4 mM; Sigma Aldrich Chemicals) and 5µl FITC-PNA (100µl/ml DPBS; Sigma Aldrich Chemicals). Samples were mixed and incubated in the dark for 15min at 38°C in an incubator (Thermoline TEI-13G, Thermoline Scientific Equipment Pty. Ltd., Smithfield, NSW, Australia). All samples were analyzed with a FACScan™ flow cytometer (Becton Dickinson) equipped with an air-cooled Argon-Ion-Laser with a wavelength of 488nm and 15mW laser power. For the measurements the filters FL-1 (530/30 nm) for green fluorescent and FL-3 (650LP nm) for red fluorescent were used. Data-analysis was performed with the Cellquest™-Software (Becton Dickinson) on a Power Mac G4-Computer (Apple Inc.). For sample analysis uniform settings were used. The debris of the egg yolk extender was gated out according to the method developed by NAGY et al. (2003). During evaluation sperm heads were divided into four groups according to their

plasma membrane integrity and acrosomal integrity: sperm with intact membrane and intact acrosome (orange staining, SYTO-17+/PNA-/PI-), membrane damaged sperm with intact or completely lost acrosome (red staining, SYTO-17-/PNA-/PI+), membrane damaged and acrosome reacted sperm (red and green staining, SYTO-17-/PNA+/PI+) and membrane intact and acrosome reacted sperm (orange and green staining, SYTO-17+/PNA+/PI-). The delimitation of sperm groups was done subjectively with the Cellquest^{TM} Software (Becton Dickinson). At least $1x10^4$ spermatozoa were analyzed per sample.

3.2.2 Freezing of boar semen following different freezing curves

3.2.2.1 Processing of semen

Semen was processed and tested for quality changes before freezing as described above. The cooling and freezing extender contained 12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol per ml.

3.2.2.2 Experimental design

After loading the semen into straws samples were submitted to either one of the following three freezing curves (Table 6) using a programmable freezer (Planer Products Kryo 10, Series III; Planer Products Ltd., Sunbury, Middlesex, UK).

Table 6: Protocol of three different freezing curves

Short freezing protocol	Medium freezing protocol	Long freezing protocol
Start temperature +4.0 °C	Start temperature +4.0°C	Start temperature +4.0!C
Cooling rate -6°C/min to -5°C	Cooling rate -3°C/min to -6°C	Cooling rate -3°C/min to -6°C
Freezing rate -40°C/min to -80°C	Holding time of 60s at -6°C	Holding time of 60s at -6°C
	Freezing rate -50°C/min to -140°C	Freezing rate -30°C/min to -140°C
Holding time of 30s at -80°C		
Freezing rate -50°C/min to -140°C		

The frozen straws were then plunged in liquid nitrogen (LN_2, -196°C) and stored until thawing.

For post-thaw semen evaluation samples were removed from LN_2 and submitted to two different thawing protocols according to the corresponding freezing curves as shown in Table 7. Quality assessment was performed as described above.

Table 7: Different thawing protocols for boar semen according to freezing methods

Short freezing protocol	Medium freezing protocol	Long freezing protocol
12s at 50°C	30s at 38°C	30s at 38°C

3.2.2.3 Semen analysis

The analyses of the thawed semen samples was conducted in the same way as described under 3.2.1.3.

3.2.2.4 Statistical analysis

Statistical analyses were performed with SIGMA STAT 2.03 for Windows (Jandel Scientific Cooperation, San Ragael, CA, USA). Effects of group treatment on the quality of the semen was analysed for normal distribution and tested with One-way ANOVA or ANOVA on Ranks. Significances were tested with the Tukey test. Data are expressed as percentages or means ± SD. Differences were considered to be significant at $P<0.05$.

3.3 Results

3.3.1 Freezing of boar semen in the presence of ROS scavengers

During the experiment 36 ejaculates were collected from four boars. The estimated motility of the samples right after collection and dilution with Androhep^{TM} was 88.9±2.1%.

Motility after transport and cooling to 15°C was 89.1 ± 6.6% motile and 62.0 ± 8.3% progressively motile spermatozoa. The mean concentration of all ejaculates was 32.7 ± 9.7 x 10^7 spermatozoa per ml. Fresh semen samples showed 6.7 ± 3.6% morphologically abnormal spermatozoa and 97.3 ± 2.3% of spermatozoa had a normal apical ridge (NAR).

Directly after thawing (0h) semen frozen with addition of Pyruvate, Catalase and Mercaptoethanol showed a significantly higher percentage of motile spermatozoa as compared to controls. Semen frozen in the presence of TROLOX showed the same motility pattern as in the control group (45.9±18.8%) and the AO-Mix group. After 6h of incubation semen frozen in the presence of Pyruvate, Catalase and Mercaptoethanol had a higher motility than the control group ($p<0.05$) although the motility was very low in both groups and irrelevant for AI.

Table 8: Percentage of motile spermatozoa exposed to a 6h thermo resistance test after thawing employing different antioxidants in the cooling and freezing extenders

%	**AO-Mix** **X̄±SD**	**Trolox** **X̄±SD**	**Control** **X̄±SD**
0 h	59.3±16.0^{bA}	54.1±17.8^{abA}	45.9±18.8^{aA}
1 h	48.6±23.9^{aA}	42.1±23.2^{aAC}	39.7±23.5^{aAC}
2 h	35.6±24.7^{aAC}	23.6±23.5^{aBC}	30.1±23.4^{aBCD}
3 h	17.1±15.8^{aBC}	13.4±12.5^{aBD}	13.2±12.6^{aBD}
6 h	3.5±3.3^{bB}	2.2±2.4^{abBD}	1.7±1.6^{aB}

a:b $p \leq 0.05$ at same time after thawing

A:B $p \leq 0.05$ within one treatment group

The percentage of morphological abnormal spermatozoa was evaluated directly after thawing and is summarized in Table 9.

Table 9: Percentage of morphologically abnormal spermatozoa directly after thawing

%	**AO-Mix** $\bar{x}$±SD	**Trolox** $\bar{x}$±SD	**Control** $\bar{x}$±SD
normal apical ridge (NAR)	34.1 ±7.4	33.6±7.8	32.8±5.5
dissolving acrosome	28.4±6.1	30.1±10.7	29.26±9.3
lost acrosome	37.6±7.4	36.3±12.6	38.0±8.0
gross morphology	66.8±6.9	66.9±7.6	67.9±5.6
other abnormalities	2.2 ± 2.2^{a}	1.5 ± 0.9^{ab}	1.4 ± 0.8^{b}

a:b $p \leq 0.05$ between treatment groups

Frozen/thawed spermatozoa were evaluated flowcytometrically at 0h and 2h after thawing. Table 10 shows the results of the triple staining.

As shown in table 10 there were no significant differences between groups, but in eleven of twelve groups the values differed significantly within a group after 2h incubation. Only the percentage of vital and acrosome reacted spermatozoa differed significantly after 2h incubation with the sample frozen with the AO-Mix showing a lower percentage than the control group (0.8±0.2% vs. 0.9±0.3%).

Table 10: Results of the flowcytometrical evaluation of membrane integrity and acrosome reaction of frozen/thawed spermatozoa applying SYTO-17/FITC-PNA/PI 0h and 2h after thawing

(%)	hours after thawing	**AO-Mix** **X̄±SD**	**Trolox** **X̄±SD**	**Control** **X̄±SD**
viable spermatozoa; intact acrosome	0h	61.8 ± 8.3^{A}	62.5 ± 9.0^{A}	61.5 ± 9.3^{A}
	2h	57.0 ± 7.7^{B}	56.9 ± 9.2^{B}	54.8 ± 10.3^{B}
viable spermatozoa; acrosome reacted	0h	0.4 ± 0.5^{A}	0.4 ± 0.2^{A}	0.4 ± 0.2^{A}
	2h	0.8 ± 0.2^{aB}	0.8 ± 2.5^{abB}	0.9 ± 0.3^{bB}
membrane damaged sperm; acrosome intact	0h	28.5 ± 6.74	28.0 ± 7.3^{A}	29.0 ± 8.1^{A}
	2h	31.5 ± 6.0	31.5 ± 7.4^{B}	33.1 ± 8.6^{B}
membrane damaged sperm; acrosome reacted	0h	9.3 ± 2.4^{A}	9.1 ± 2.5^{A}	9.1 ± 1.9^{A}
	2h	10.7 ± 2.6^{B}	10.8 ± 2.5^{B}	11.2 ± 2.5^{B}

a:b p≤0:05 at same time after thawing between treatment groups

A:B p≤0:05 within same treatment groups at different times after thawing

3.3.2 Quality assessment of frozen/thawed boar semen against individual freezing speeds

During the experiment six ejaculates were obtained from four boars. Table 11 shows the motility results directly after collection, and dilution, and after cooling to 15°C as evaluated with a computer assisted sperm analyzing (CASA) system.

Table 11: Motility of fresh semen samples directly after collection and dilution and after cooling to 15°C as analyzed with CASA

(%)		**Boar A** **x̄±SD**	**Boar B** **x̄±SD**	**Boar C** **x̄±SD**	**Boar D** **x̄±SD**
Motility directly after collection and dilution (%)		88.0±4.5	90.0±0.0	89.2±2.0	90.0±0.0
Motility after cooling to 15°C (CASA) (%)	gross motility	88.8±4.0	93.7±2.0	92.0±2.5	93.7±3.0
	progressive motility	67.3±5.8	76.7±2.3	59.0±5.2	66.8±5.2

The concentration of the semen samples (n=6) from four boars after 1:2 dilution with Androhep™ as determined with a haemocytometer was 360.8±104.9 x 10^6 per ml for boar A, 514.2±161.2 x 10^6 per ml for boar B, 349.17±118.3 x 10^6 per ml for boar C and 535.3±117.8 x 10^6 per ml for boar D. Table 12 summarizes the results of the morphological evaluation of the fresh semen samples.

Table 12: Percentage of morphologically abnormal spermatozoa after Glutaraldehyd fixation (6 ejaculates from 4 four boars each) – Fresh semen samples

(%)	**Boar A** **x̄±SD**	**Boar B** **x̄±SD**	**Boar C** **x̄±SD**	**Boar D** **x̄±SD**	**Total** **x̄±SD**
normal apical ridge (NAR)	96.6±0.6	99.7±0.4	97.6±0.9	98.7±0.5	98.2±1.3
dissolving acrosomes	1.9±0.6	0.1±0.6	0.9±0.9	0.7±0.6	1.0±0.8
lost acrosomes	1.4±0.6	0.2±0.4	1.3±0.9	0.6±0.5	0.9±0.7
other abnormalities	12.1±4.6	11.1±7.8	4.5±1.8	3.0±1.5	7.7±5.9
gross morphology	15.4±4.6	11.4±8.0	6.9±1.2	4.4±1.4	9.5±6.2

Directly after thawing the motility of the frozen/thawed sperm samples was evaluated in a thermo-resistance-test. Table 13-16 show the motility of the different freezing protocols for each boar.

There was no significant difference for boar A during the thermo resistance test in the percentage of motile spermatozoa between samples frozen in either one of the respective freezing protocols. However, there was a significant motility decrease in all treatment groups during the incubation period.

Table 13: Sperm motility of Boar A in a thermo-resistance-test of spermatozoa frozen and thawed according to one of three different freezing protocols

(%)	**Short** $\bar{x}$±SD	**Medium** $\bar{x}$±SD	**Long** $\bar{x}$±SD
0 h	67.5 ± 12.9^{A}	65.0 ± 10.0^{A}	65.0 ± 8.4^{A}
1 h	52.5 ± 9.4^{A}	59.2 ± 11.1^{A}	57.5 ± 8.2^{A}
2 h	49.2 ± 8.6^{B}	54.2 ± 14.3^{A}	53.3 ± 10.8^{A}
3 h	27.1 ± 12.1^{B}	36.7 ± 16.3^{B}	39.2 ± 12.4^{B}
6h	3.5 ± 5.7^{B}	6.2 ± 5.3^{B}	6.0 ± 4.7^{B}

A:B:C $p\leq0.001$ within one treatment group

There was no significant difference for boar B during the thermo resistance test in the percentage of motile spermatozoa between samples frozen in either one of the respective freezing protocols, but the motility decreased significantly in all during groups over time.

Table 14: Sperm motility of Boar B in a thermo-resistance-test of spermatozoa frozen and thawed according to three different freezing curves

(%)	**Short** **x̄±SD**	**Medium** **x̄±SD**	**Long** **x̄±SD**
0 h	52.5±17.8^{A}	57.5±12.9^{A}	57.5±23.2^{A}
1 h	55.0±17.0^{A}	48.3±24.0^{A}	49.2±17.2^{A}
2 h	37.5±16.34^{A}	32.5±22.1^{B}	33.3±17.8^{A}
3 h	13.0±8.0^{B}	13.8±7.2^{C}	9.7±5.0^{B}
6h	1.7±2.0^{B}	0.5±0.6^{D}	1.2±1.9^{B}

A:B:C:D $p \leq 0.001$ within one treatment group

Semen of boar C frozen with the ”long” freezing curve showed a significant better sperm motility directly after thawing than samples frozen with the short curve (66.7±8.2% vs. 43.3±10.8%). The groups frozen in different freezing protocols did not differ significantly from one to six hours after thawing and start of incubation, although the percentage of motile spermatozoa was always higher for the sample frozen in the long freezing curve. During the incubation the motility decreased significantly over time.

Table 15: Sperm motility of Boar C during a thermo-resistance-test of spermatozoa frozen and thawed according to three different freezing curves

(%)	**Short** **x̄±SD**	**Medium** **x̄±SD**	**Long** **x̄±SD**
0 h	43.3±10.8^{aA}	51. 7±18.4^{A}	66. 7±8.2^{bA}
1 h	32.5±8.2^{A}	47.5±15.7^{A}	57.5±11.3^{A}
2 h	21. 7±15.7^{B}	35.8±16. 6^{A}	52.5±9.4^{A}
3 h	11.7±7.5^{B}	28.3±16.6^{A}	37.5±12.1^{B}
6h	1.2±1.9^{B}	2.0±2.0^{B}	6.8±4.8^{B}

a:b $p \leq 0.05$ at same time after thawing between treatment groups

A:B $p \leq 0.05$ within one treatment group

There was no significant difference for boar D during the thermo resistance test in the percentage of motile spermatozoa between samples frozen in either one of the respective freezing protocols but motility decreased significantly over time.

Table 16: Sperm motility of Boar D in a thermo-resistance-test of spermatozoa frozen and thawed according to three different freezing curves

(%)	**Short** $\bar{x}$±SD	**Medium** $\bar{x}$±SD	**Long** $\bar{x}$±SD
0 h	70.8±6.7^{A}	66. 7±9.3^{A}	61. 7±8.2^{A}
1 h	60.8±10.7^{B}	65.0±0.0^{A}	59.2±8.6^{A}
2 h	46. 7±8.8^{C}	45.0±20.3^{B}	42.5±13.7^{B}
3 h	25.8±9.2^{D}	34.2±18.0^{C}	23.3±13.7^{B}
6h	2.2±2.2^{E}	3.7±3. 6^{D}	3.5±3.3^{B}

A:B:C:D:E $p \leq 0.05$ within one treatment group

Table 17 shows the mean motility of all boars during the thermo resistances test in the percentage of motile spermatozoa between samples frozen in either one of the respective freezing protocols. There was no significant difference between the groups but the motility decreased significantly over time.

Table 17: Mean motility of all boars during the thermo resistances test in the percentage of motile spermatozoa between samples frozen in either one of the respective freezing protocols

(%)	**Short** $\bar{x}$±SD	**Medium** $\bar{x}$±SD	**Long** $\bar{x}$±SD
0 h	58.5±16.5^{A}	60.2±13.8^{A}	62.7±13.2^{A}
1 h	50.2±15.5^{A}	55.0±16.2^{A}	55.8±11.8^{A}
2 h	38.8±16.3^{A}	41.9±19.3^{A}	45.4±15.0^{B}
3 h	19.5±11.4^{B}	28.3±16.8^{B}	27.4±16.1^{B}
6h	2.1±3.3^{C}	3.1±3.8^{C}	4.4±4.3^{B}

A:B:C $p \leq 0.001$ within one treatment group

There were no significant differences between the groups in the percentage of morphological abnormal spermatozoa for boar A.

Table 18: Percentage of morphological abnormal spermatozoa of Boar A directly after thawing

(%)	**Short** $\bar{x}$±SD	**Medium** $\bar{x}$±SD	**Long** $\bar{x}$±SD
normal apical ridge (NAR)	34.8±6.7	35.5±9.8	25.7±10.0
dissolving acrosomes	26.2±6.7	23.0±7.2	29.9±11.8
lost acrosomes	39.0±6.6	41.5±9.8	44.4±9.9
other abnormalities	2.0±1.0	1.7±0.6	1.7±1.2
gross morphology	67.6±6.6	66.2±9.9	75.9±10.0

Data differed not significantly

There were no significant differences between the groups in the percentage of morphological abnormal spermatozoa for boar B.

Table 19: Percentage of morphologically abnormal spermatozoa of Boar B directly after thawing

(%)	**Short** $\bar{x}$±SD	**Medium** $\bar{x}$±SD	**Long** $\bar{x}$±SD
normal apical ridge (NAR)	26.7±8.5	26.5±7.66	24.5±5.8
dissolving acrosomes	32.1±9.5	37.0±7.7	32.9±5.7
lost acrosomes	41.2±8.2	36.5±11.3	42.6±6.9
other abnormalities	4.0±0.0	1.3±0.6	1.0±0.0
gross morphology	77.3±8.3	74.8±7.08	76.6±6.1

Data differed not significantly

There were no significant differences between the groups in the percentage of morphological abnormal spermatozoa for boar C.

Table 20: Percentage of morphologically abnormal spermatozoa of Boar C directly after thawing

(%)	**Short** **x̄±SD**	**Medium** **x̄±SD**	**Long** **x̄±SD**
normal apical ridge (NAR)	24.5±9.3	25.8±5.9	31.7±9.3
dissolving acrosomes	29.9±7.3	23.0±7.0	31,2±10.3
lost acrosomes	45.6±11.6	51.2±9.8	37.6±8.9
other abnormalities	1.0±0.0	1.0±0.0	1.0±0.0
gross morphology	76.4±9.2	75.1±5.8	69.8±9.4

not significantly different

There were no significant differences between the groups in the percentage of morphological abnormal spermatozoa for boar D.

Table 21: Percentage of morphologically abnormal spermatozoa of Boar D directly after thawing

(%)	**Short** **x̄±SD**	**Medium** **x̄±SD**	**Long** **x̄±SD**
normal apical ridge (NAR)	32.3±7.2	32.5±6.6	28.8±5.9
dissolving acrosomes	27.3±7.2	31.9±6.6	24.7±5.9
lost acrosomes	40.4±6.3	35.6±7.7	46.5±5.6
other abnormalities	1.0±0.0	3.0±0.0	1.5±0.7
gross morphology	68.7±7.6	70.5±7.2	72.7±6.5

not significantly different

Table 22 summarizes the morphological results for all boars directly after thawing. There were no significant differences between the treatment groups of all boars.

Table 22: Percentage of morphologically abnormal spermatozoa of all boars directly after thawing

(%)	**Short** $\bar{x}$±SD	**Medium** $\bar{x}$±SD	**Long** $\bar{x}$±SD
normal apical ridge (NAR)	29.6±8.1	30.1±8.3	27.5±7.9
dissolving acrosomes	29.3±5.9	35.0±8.6	33.6±6.2
lost acrosomes	41.1±8.6	34.9±8.3	38.9±7.9
other abnormalities	1.6±1.0	1.6±0.7	1.4±0.7
gross morphology	71.9±8.5	71.5±8.2	73.9±8.1

not significantly different

The acrosome reaction and membrane integrity of the frozen/thawed spermatozoa was flowcytometrically analyzed applying a triple staining method with SYTO-17/FITC-PNA/PI. The sperm samples were evaluated at 0h and 2h after thawing. Table 23-26 show the results of the evaluation for each boar.

There was no significant difference for boar A between freezing treatments neither in the percentage of spermatozoa with intact membranes nor with acrosome reaction .

Table 23: Flow cytometrical evaluation of Boar A sperm membrane integrity and acrosome reaction of frozen/thawed spermatozoa applying SYTO-17/FITC-PNA/PI 0h and 2h after thawing

(%)	hours after thawing	**Short** **x̄±SD**	**Medium** **x̄±SD**	**Long** **x̄±SD**
vital spermatozoa; intact acrosome	0h	45.7±5.8	40.9±12.5	51.4±6.5
	2h	40.5±5.6	37.2±8.6	43.0±6.5
vital spermatozoa; acrosome reacted	0h	0.9±0.3	0.9±0.5	0.8±0.4
	2h	1.1±0.3	1.3±0.3	1.0±0.2
membrane damaged sperm; acrosome intact	0h	36.9±4.7	41.6±9.0	32.9±5.6
	2h	38.1±3.7	41.6±7.6	37.9±4.9
membrane damaged sperm; acrosome reacted	0h	16.5±2.9	16.6±4.6	15.0±2.0
	2h	20.4±4.4	19.9±2.1	18.1±2.7

no significant differences

The percentage of viable, acrosome reacted spermatozoa and the percentage of membrane damaged and acrosome reacted spermatozoa of boar B was significantly lower in the sample frozen following the medium timed protocol than following the short and long protocol (Table 24).

Table 24: Flow cytometrical evaluation of Boar B sperm membrane integrity and acrosome reaction of frozen/thawed spermatozoa applying SYTO-17/FITC-PNA/PI 0h and 2h after thawing

(%)	hours after thawing	**Short** $\bar{x}$±SD	**Medium** $\bar{x}$±SD	**Long** $\bar{x}$±SD
viable spermatozoa; intact acrosome	0h	49.5±7.5	56.2±5.8	49.0±11.59
	2h	47.7±7.6	47.0±4.2	44.5±4.8
viable spermatozoa; acrosome reacted	0h	2.2±0.7^{aA}	1.2±0.3^{b}	4.5±7.1^{b}
	2h	2.2±1.0^{B}	1.5±0.3	1.3±0.4
membrane damaged spermatozoa;	0h	31.6±9.9	31.9±4.6	31.1±4.9
	2h	29.8±9.3	35.1±4.0	38.2±4.4
membrane damaged spermatozoa;	0h	16.6±3.4^{aA}	10.8±2.4^{bA}	15.4±8.08^{ac}
	2h	20.3±6.8	16.5±3.6^{B}	16.0±2.6

a:b:c $p \leq 0.05$ at same time after thawing between treatment groups

A:B $p \leq 0.001$ within one treatment group

For boar C no significant differences between the three freezing protocols were seen. However, the percentage of viable, acrosome reacted spermatozoa and the percentage of membrane damaged and acrosome reacted spermatozoa was significantly higher ($p \leq 0.05$) after two hours of incubation of the sample frozen in the short freezing curve.

Table 25: Flow cytometrical evaluation of Boar C sperm membrane integrity and acrosome reaction of frozen/thawed spermatozoa applying SYTO-17/FITC-PNA/PI 0h and 2h after thawing

(%)	hours after thawing	**Short** **$\bar{x}$±SD**	**Medium** **$\bar{x}$±SD**	**Long** **$\bar{x}$±SD**
viable spermatozoa; intact acrosome	0h	61.8±7.5	63.5±6.5	51.07±19.5
	2h	57.4±3.4	54.3±5.5	55.7±6.0
viable spermatozoa; acrosome reacted	0h	0.6±0.5^{A}	0.7±0.2	1.0±0.5
	2h	1.7±0.6^{B}	1.1±0.2	1.1±0.2
membrane damaged spermatozoa;	0h	29.8±8.5	26.4±4.8	35.8±16.7
	2h	25.9±5.8	31.5±3.7	28.7±5.9
membrane damaged spermatozoa;	0h	7.8±4.8^{A}	9.3±2.1	12.3±4.2
	2h	15.0±2.5^{B}	13.1±2.5	15.5±2.0

a:b:c $p \leq 0.05$ at same time after thawing between treatment groups

A:B $p \leq 0.001$ within one treatment group

For boar D there were no significant differences between the three groups but a part of the values differed significantly after two hours of incubation as presented in Table 26.

Table 26: Flow cytometrical evaluation of Boar D sperm membrane integrity and acrosome reaction of frozen/thawed spermatozoa applying SYTO-17/FITC-PNA/PI 0h and 2h after thawing

(%)	hours after thawing	**Short** **X±SD**	**Medium** **X±SD**	**Long** **X±SD**
viable spermatozoa; intact acrosome	0h	61.4±4.7	58.95±3.76^{A}	55.3±11.1
	2h	51.9±3.2	45.4±3.9^{B}	48.8±8.9
viable spermatozoa; acrosome reacted	0h	1.3±0.7^{A}	0.7±0.2^{A}	0.7±0.6
	2h	2.4±1.1^{B}	1.6±0.2^{B}	1.2±0.3
membrane damaged spermatozoa;	0h	22.4±6.4	29.0±2.4	31.5±8.4
	2h	24.1±5.5	33.1±3.0	33.4±8.5
membrane damaged spermatozoa;	0h	15.0±2.2^{A}	11.5±2.8^{A}	12.5±3.3
	2h	21.6±3.1^{B}	19.4±2.2^{B}	16.7±3.3

a:b:c $p \leq 0{,}05$ at same time after thawing between treatment groups

A:B $p \leq 0.001$ within one treatment group

For the mean of all boars there were no significant differences between the treatment groups, whereas significant differences could be detected in some cases after two hours of incubation (see Table 27).

Table 27: Flow cytometrical evaluation of sperm membrane integrity and acrosome reaction of frozen/thawed spermatozoa applying SYTO-17/FITC-PNA/PI 0h and 2h after thawing for all boars

(%)	hours after thawing	**Short** **X±SD**	**Medium** **X±SD**	**Long** **X±SD**
viable spermatozoa; intact acrosome	0h	54.6±9.5^{A}	54.9±11.3^{A}	51.7±12.4
	2h	49,4±8,0^{B}	46,1±8,2^{B}	48,0±8,0
viable spermatozoa; acrosome reacted	0h	1.3±0.8^{A}	0,9±0,4^{A}	1,7±3,7
	2h	1,8±0,9^{B}	1,4±0,3^{B}	1,2±0,3
membrane damaged spermatozoa;	0h	30,1±8,9	32,2±7,9^{A}	32,8±9,2
	2h	29,5±8,1	35,3±6,0^{B}	34,5±6,9
membrane damaged spermatozoa;	0h	14,0±4,9^{A}	12,0±4,0^{A}	13,8±4,8^{A}
	2h	19,3±5,0^{B}	17,2±3,7^{B}	16,3±2,8^{B}

A:B $p \leq 0.05$ within one treatment group

3.4 Discussion

Porcine production worldwide would benefit from high fertile frozen/thawed semen usable for artificial insemination as semen could then easily be exchanged among animal breeding companies. Furthermore, gene banks for preservation of high value genetics would be possible. Up until now, artificial insemination with frozen/thawed boar semen has not reached the same fertility rates as insemination with fresh or cooled semen.

The aim of the present study was to use two approaches to improve the quality of frozen thawed boar semen.

The **first study** showed that the addition of antioxidants to cooling and freezing media can improve the motility after thawing of boar spermatozoa (Table 8). The presence of Catalase, Na-Pyruvate and Mercaptoethanol in cooling and freezing media significantly improved the motility after thawing as compared to control semen without additives. Addition of the vitamin E analogue Trolox did not cause a significant increase in motility. Other reports showed ambivalent results after addition of antioxidants (Askari et al. 1994a, Maxwell and Stojanov 1996, Upreti et al. 1997, Pena et al. 2004b, 2003b, Ball et al. 2001) as the observed effects of antioxidants depended on the kind of substance used and the dose applied. However, it is generally accepted that cryo-preservation induces the formation of reactive oxygen species (ROS) (Watson 2000). Oxygen radicals are known to cause a decrease in motility, induction of capacitation and damage of membrane systems by lipid peroxidation (De Lamirande and Gagnon 1992b, Ichikawa et al. 1999, Oehninger et al. 1995). This is due to the high content of unsaturated fatty acids, which are prone to ROS induced damage (Cerolini et al. 2001). The present results of a supportive action of antioxidants agree with other reports employing antioxidants during the freezing process (Pena et al. 2004b, Cerolini et al. 2001). The effect of the AO-Mix confirms also similar reports on bull semen preservation where Sexcess® has been used for the first time with sex-sorted frozen spermatozoa (Klinc 2005). But the results shown herein did not indicate any improvement to protect the normal apical ridges (NAR) nor the percentage of membrane intact spermatozoa in general. This is contrary to the studies with antioxidants in bull semen (Klinc 2005) where they improved both quality criteria. Pena et al. (2003b) has found that Trolox caused,

beside its beneficial effect on motility, a higher mitochondrial membrane potential in boar spermatozoa. Cummins et al. (1994) indicated that mitochondria are likely to be the most sensitive part of spermatozoa towards freezing and thawing. It is possible that a mixture of Pyruvate, Catalase and Mercaptoethanol protects the energy metabolism in frozen spermatozoa and improves post-thaw sperm motility. However, they do not sufficiently protect membrane integrity due to the high content of polyunsaturated fatty acids in porcine spermatozoa.

In the **second study** only little differences in sperm quality could be demonstrated after thawing of boar sperm when using different freezing protocols for individual boars. Only one boar showed significantly better motility after freezing spermatozoa in a prolonged freezing protocol. The other three animals did not show any significant preferences for any protocol. A similar result has been found for acrosome and membrane integrity for all boars.

Spermatozoa are exposed to physical and chemical stress during the process of cooling and freezing, caused by ice formation and changing osmotic conditions during cooling and freezing. Sperm survival after freezing and thawing seems to depend on basic properties of the sperm plasma membrane like biochemical composition, thermal behaviour, osmotic resistance and physical stresses during sperm processing (Hammerstedt et al. 1990, De Leeuw et al. 1991). Sperm recovery is altered by cooling and freezing rates (Fiser and Fairfull 1990, Bwanga et al. 1991a). However, there are complex interactions between cooling rate, glycerol concentration and warming rates causing a different outcome in sperm quality after thawing (Fiser et al. 1991, Fiser et al. 1993). Variation in individual males can overshadow these factors. These factors can be minimized by selecting animals on the basis of cryo-resistance of their semen samples (Almlid et al. 1989b, Berger and Fischerleitner 1992). This could explain the lack of differences between the individual freezing protocols for the four boars used in this experiment. Medrano (1998) supported the view that considerable individual variation between boars exist. In their work post thawing motility varied between 1 and 38% and the plasma membrane integrity varied between 4 and 36%. The two parameter were not correlated. Additionally, Holt et al. (2005) showed that post-thaw sperm survival for any individual boar showed little change in response to manipulation of cooling rates. Additionally, while inter-ejaculate variance in post-thaw sperm survival was

detected, it was significantly outweighed by the inter-boar variance. These findings agree with those of Roca et al. (2006a), where 70% of total variability in cryo-survival of boar semen could be explained by boar effects. Thus, pre-selection of boars for freezeability is a reliable way of assuring a higher post thaw semen quality.

From these findings it can be concluded that antioxidants added to boar semen freezing extender significantly improve the post-thaw sperm motility in particular. Different rates for freezing spermatozoa of individual boars may have only limited effects on post-thaw semen quality if the boars have been previously selected for cryo-resistance.

4. CHAPTER 2: Improvement of sex-sorted fresh and frozen-thawed boar spermatozoa and their subsequent insemination efficiency after supplementation of the extender with antioxidants

4.1 Introduction

Gender pre-determination in mammalian offspring by means of flow cytometrical sperm sorting was established first in rabbit spermatozoa (Johnson et al. 1989) and two years later in swine (Johnson 1991). Until now, sperm sexing technology has not been implemented in swine production, although the increased use of AI in pigs demands to alter sex ratio, particularly with genetic companies seeking to produce large numbers of seed stock gilts in multiplier herds. The reason for this is the still limited sperm output even with fast high speed flow cytometers that allow to sort sperm populations of high purity at around 15 million spermatozoa per sex and hour (Johnson et al. 2005b). Sex sorted fresh spermatozoa have been used in sows with non-surgical deep intrauterine insemination (DUI) (Martinez et al. 2001, Rath et al. 2003a, Grossfeld et al. 2005). However, due to the limited fertilizing ability of sex sorted fresh boar spermatozoa the usage requires to have potential sows in close vicinity to the laboratory and to inseminate them shortly before ovulation. These limitation can be overcome if sex sorted spermatozoa could be stored for a longer period in either fresh or frozen state. The long-time storage of sex-sorted semen would furthermore ease the production of gender pre-selected piglets even in herds far away from the sorting laboratory.

Until now no piglets have been produced after non-surgical AI with sex-sorted frozen/thawed spermatozoa. In opposite, in cattle a recently new developed post sorting technology (Sexcess®) has significantly improved the fertility of sex sorted frozen spermatozoa (Klinc 2005). Related to this technique, semen additives have been tested in boar semen and improved the post-thaw quality of non-sorted frozen/thawed boar semen significantly (see Chapter I).

In this study, three different trials tested the effect of antioxidants on the quality of liquid stored semen as well as frozen sex-sorted spermatozoa. Additionally, it was evaluated whether piglets can be produced after surgical insemination with sorted frozen/thawed spermatozoa.

4.2 Material and Methods

4.2.1 Experiment 1: Improvement of sex sorted fresh boar spermatozoa using Androhep™ supplemented with different antioxidants

4.2.1.1 Semen processing

Ejaculates were collected from four boars of the AI-Centre Mariensee using the gloved hand method. Fifty millilitre of the sperm-rich fraction were collected into collection bags (Minitüb, Tiefenbach, Germany) fixed in a thermos receptacle. The Bulbourethral-gland secretion was separated by a filter included in the collection bag. Directly after collection semen was divided into two parts. One part was diluted with pre-warmed (38°C) Androhep™ semen extender (Minitüb, Tiefenbach, Germany) (1:1, v/v) containing 24.6 IU/ml Catalase, 2 mMol Na-Pyruvate and 106 µMol Mercaptoethanol (all from Sigma, St.Louis, MO, USA) per ml resulting in a final concentration of 12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol per ml in the diluted semen. The other part of the ejaculate was diluted in the same way, but without additives. Semen was then transported in an isolated container to the laboratory. Spermatozoa were evaluated for motility, concentration and morphology according to the methods described by Krause (1966). Sperm motility was evaluated under a phase contrast microscope (Olympus BX 60, Olympus) supplemented with a heating stage (38°C) at 100x magnification. The concentration of the semen was determined in a Thoma-Counting-chamber new (Herenz Medizinalbedarf, Hamburg, Germany). Additionally an aliquot of 50µl of semen was fixed in 500µl of Hancock-solution and the morphological status was examined at 1000 x magnification under oil immersion. At least 200 spermatozoa were counted.

4.2.1.2 Semen processing for flow cytometrical sorting

Sperm samples were diluted with Androhep™ to 100 x 10^6 per ml. For flow cytometrical sorting 7.5-10µl Hoechst 33342 dye (Sigma, St.Louis, MO, USA) from a 5mg/ml stock-solution were added. Samples were incubated for 75 minutes at 38°C. Thereafter, 1µl of food dye (FD&C #40, Warner Jenkins Company Inc., St. Louis, MO, USA) was added from a 25 mg/ml stock-solution to gate out membrane-damaged spermatozoa in the flow cytometer. Until sorting the samples were stored at room-temperature in the dark and were filtered in a nylon-filter with a pore diameter of 51µm (Reichelt Chemietechnik, Heidelberg) shortly

before sorting. Flow cytometrical sorting was conducted according to the method developed by Johnson and Welch (1999) with a "High speed cell Sorter" (MoFlo; DakoCytomation, Fort Collins, CO, USA). The flow cytometer was equipped with a 5W Argon-laser with an optical power of 200mW. The wave length of the laser was 351 to 364 nm. Dulbecco's Phosphate Buffered Saline (DPBS) served as sheath fluid. The sheath fluid contained 12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol. The flow cytometer operated with a pressure of 3.9 bar at a speed of 27,000 events per second. The sort-rate varied from 2,700 to 3,000 spermatozoa per second. Sorted samples were collected in 10ml centrifuge tubes (Sarstedt, Nümbrecht), containing 0.5ml of a 2% TEST egg yolk-solution supplemented with 10% seminal plasma. About 8 x 10^6 spermatozoa were sorted per collection tube and were then centrifuged for 20min. at 850 x g. The supernatant was discharged and the sperm pellet was re-suspended with Androhep™ containing 12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol. Sorting purity was reanalysed using the Gauss7 program for curve fitting distribution (Johnson 1991).

4.2.1.3 Experimental design

Unsorted semen

Aliquots of 5 ml diluted semen from both groups with and without antioxidant supplementation were filled in 10ml centrifuge tubes (Sarstedt, Nümbrecht) and stored at 15°C. After 0, 24, and 120h of storage, an aliquot of 500µl was taken and placed into an Eppendorf-cup on a heated aluminium-block (38°C). The motility was recorded after 15min and 2h. Additionally after 120h of storage at 15°C the morphological status of the samples was recorded.

Sex sorted semen

After sorting and subsequent centrifugation of collection tubes, supernatant was removed and sex-sorted samples were divided into two groups and re-suspended with 80µl of Androhep™ containing 12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol or no additives. The samples were subsequently stored at 15°C. After 0, 24 and 120h of storage an aliquot of 20µl was taken from the stored samples and was warmed in an Eppendorf-cup at 38°C. The motility was recorded after 15min and 2 hours of incubation. Additionally, the morphological status of spermatozoa stored at 15°C was recorded after 120h of storage.

4.2.2 Experiment 2: Improvement of sex sorted frozen boar spermatozoa using cooling and freezing media supplemented with different antioxidants

4.2.2.1 Semen processing

The semen was obtained and processed as described in experiment 1.

4.2.2.2 Flow cytometrical analysis of acrosome reaction and membrane integrity of fresh spermatozoa

The degree of acrosome reaction and membrane integrity of fresh spermatozoa was flow cytometrically analyzed applying a triple staining method with SYTO-17/FITC-PNA/PI (Garner et al. 1999, Krienke 2003). At least 10,000 spermatozoa were analyzed per sample. For staining, semen samples were diluted to a final concentration of $5x10^6$ spermatozoa/ml with Androhep^{TM}. Then samples were stained with SYTO-17^{TM} (Molecular Probes, Invitrogen, Karlsruhe, Germany), FITC-PNA and Propidium Iodide 95-98% (both Sigma-Aldrich Chemicals, St. Louis, USA). Five hundred micro litre of diluted semen were stained with 2µl SYTO-17TM (0,5mM), 4µl PI (2,4 mM) and 5µl FITC-PNA (100µl/ml DPBS). The samples were mixed and incubated for 15min at 38°C in an incubator in the dark. Labelled samples were analyzed with a FACScan^{TM} flow cytometer (Becton Dickinson) equipped with an air-cooled Argon-Ion-Laser with a wave length of 488nm and 15mW laser power. For the measurements filters were used at 530/30 nm (FL-1) for green fluorescence and at 650LP nm (FL-3) for red fluorescence. Data-acquisition was performed with a Power Mac G4-Computer (Apple Inc.) and the Cellquest^{TM} Software (Becton Dickinson). For data analysis of samples uniform settings were used. During evaluation the sperm heads were divided into four groups according to their plasma membrane and acrosomal integrity: Sperm with intact membrane and intact acrosome (orange staining, SYTO-17+/PNA-/PI-), membrane damaged sperm with intact or complete lost acrosome (red staining, SYTO-17-/PNA-/PI+), membrane damaged and acrosome reacted sperm (red and green staining, SYTO-17-/PNA+/PI+) and membrane intact and acrosome reacted sperm (orange and green staining, SYTO-17+/PNA+/PI-). The delimitation of the sperm groups was done subjectively with the WinMDI-Software 2.8 (Trotter 1998).

4.2.2.3 Semen processing of flow cytometrical sex sorting

Flow cytometrical sorting was performed as described in experiment 1.

4.2.2.4 Experimental design

Sperm cryo-preservation was performed according to a modified Westendorf-Protocol (Westendorf et al. 1975).

4.2.2.4.1 Unsorted semen

Sperm samples were divided into two groups and were diluted with Androhep^{TM} to a concentration of either 40 x 10^6 or 80 x 10^6 spermatozoa per ml. Four millilitres of diluted semen of each concentration were filled in 10ml centrifugation tubes (Sarstedt, Nümbrecht) and cooled in a water coated tube to 15°C over a period of one hour. The samples were then centrifuged in a cooling centrifuge (Minifuge 2, Heraeus-Christ, Osterode, Germany) at 15°C for 10min at 800 x g. After centrifugation the supernatant was discarded. Then, the sperm pellets were slowly resuspended with 4ml of cooling extender (20% egg yolk, 80% Lactose-solution, v/v) containing either the low (12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol) or high (24.6 IU/ml Catalase, 2 mMol Na-Pyruvate and 106 µMol Mercaptoethanol) concentration of antioxidants per ml or no additives. The sperm samples were then cooled in an incubator to 5°C over a period of 2h and were transferred into a cooling room for further processing. Then 2ml of freezing extender [92.5% cooling extender, 6% Glycerol (Sigma, St. Louis, MO, USA) and 1.5% Equex-STM (Nova Chemicals, Situate, MA, USA) , v/v] were slowly added to each sperm sample. The freezing extender contained the same amount of antioxidants as the cooling extender. Semen was filled in 0.25ml plastic-straws (Minitüb, Tiefenbach, Germany). Each straw contained either 8 or 16 x 10^6 spermatozoa. Freezing of the samples was performed in a closed Styrofoam box (30 cm x 40 cm x 85 cm = high x width x length). Briefly, straws were placed on a metal holder in nitrogen vapour 4 cm above liquid nitrogen level for 20 minutes. Frozen samples were then plunged into liquid nitrogen and kept in a storage container until analysis.

4.2.2.4.2 Sex-sorted semen

After sorting and centrifugation the sperm pellet (30µl) with sexed spermatozoa was re-suspended with either 45µl or 120µl cooling extender (20% egg yolk, 80% Lactose-solution, v/v) containing either the low (12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol) or high (24.6 IU/ml Catalase, 2 mMol Na-Pyruvate and 106 µMol

Mercaptoethanol) concentration of antioxidants per ml or no additives. The sperm samples were placed in a water coated tube and cooled in a cooling room to 5°C over period of 2h. The minimum storage time in the cooling room was 2 hours, the maximum time 4 hours. After cooling to 5°C two of the sperm samples resuspended with 45 µl of cooling extender were put together in one tube. Thereafter, 75 µl freezing extender [92.5% cooling extender, 6% Glycerol (Sigma, St. Louis, MO, USA) and 1.5% Equex-STM (Nova Chemicals, Situate, MA, USA) , v/v] containing the same amount of antioxidants as the cooling extender were added to all sperm samples and the semen was filled in 0.25ml plastic-straws (Minitüb, Tiefenbach, Germany). Each straw contained either 8 or 16 x 10^6 spermatozoa. Freezing of the samples was performed in a closed Styrofoam box as described above.
Table 28 summarizes the treatment groups of experiment 2

Table 28: Treatment groups for cryo-preservation of sex-sorted semen

Treatment	**unsexed semen**	**sex-sorted spermatozoa**	**Antioxidants**	**Sperm per straw x 10^6**
Control-1	X		none	8
Control-2	X		low	8
Control-3	X		high	8
Control-4	X		low	16
Sexed-1		X	none	8
Sexed-2		X	low	8
Sexed-3		X	high	8
Sexed-4		X	none	16
Sexed-5		X	low	16
Sexed-6		X	high	16

4.2.2.5 Analysis of frozen thawed spermatozoa

Straws were thawed at 38°C for 30 sec. and were emptied in 1.5ml Eppendorf cups placed on a heated aluminium-block (38°C). Androhep™ (400µl) was added to each Eppendorf cup and sperm samples were kept at 38°C until evaluation. The motility of the frozen/thawed semen samples was analyzed 10min, 1h, 2h, 3h and 6h after thawing under a phase-contrast microscope (Olympus BX 60, Olympus) equipped with a heating plate (Minitüb, Tiefenbach, Germany) at 38°C. Two drops and at least three fields per drop were analysed in each sample at 100x magnification.

4.2.2.6 Analysis of morphologically abnormal spermatozoa

For analysis of frozen/thawed spermatozoa an aliquot of 20µl thawed spermatozoa was put in an Eppendorf tube and mixed with 40µl Hancock-Solution. Morphological abnormalities and acrosome integrity were analysed under a phase-contrast microscope (Olympus BX 60, Olympus) at 1000x magnification after fixation. At least 100 spermatozoa were examined in the frozen/thawed samples.

4.2.2.7 Flow-cytometrical analysis of acrosome reaction and membrane integrity of frozen/thawed spermatozoa (SYTO-17, FITC-PNA, PI)

The acrosome reaction and membrane integrity of the frozen/thawed spermatozoa was flow cytometrically analyzed as described above. At least 3000 spermatozoa were analyzed per sample. The debris of the egg yolk extender was gated out according to the method developed by Nagy et al. (2003).

4.2.3 Experiment 3: Tubal insemination of sex-sorted frozen/thawed boar spermatozoa

In the third experiment boar semen was collected, sex-sorted and frozen/thawed as described above. Additionally control semen with 2 million spermatozoa per straw containing 12.3 IU/ml Catalase, 1 mMol Na-Pyruvate and 53 µMol Mercaptoethanol per ml was prepared. For tubal insemination 2 million spermatozoa per straw were used.

4.2.3.1 Surgical insemination procedure

Six prepuberal gilts (German Landrace) housed in individual crates were inseminated surgically into the oviduct. Therefore gilts were synchronized with 1500 IU PMSG i.m. and 500 IU hCG administered i.m. 72h later. Surgical insemination into the oviduct was performed 38 h after hCG treatment short before ovulation. Gilts were anesthetized with 8 ml Stresnil and 25ml Trapanal. They were prepared for surgery in dorsal recumbence, and the uterine horns and oviducts were exposed by mid-ventral laparotomy. Frozen sex-sorted and control sperm samples were thawed directly before insemination for 12 sec at 50°C in a water bath and motility was recorded. Per oviduct the contents of one straw was used for insemination. The straws were connected to a blunt 18g needle that was inserted into the tip of the uterine horn, guided towards the utero- tubal junction (UTJ) and semen was released into the oviductal ampoule. The procedure was repeated in the opposite horn, and the genital

tract was replaced into the abdominal cavity. Surgical closure was performed with Safil C, no. 7 metric (B/BRAUN, Aesculap, Melsungen, Germany).

To evaluate the number of fertilized oocytes the animals were slaughtered 48 hours later, the genital tract was extracted and transported in an isolated container into the laboratory. The oviducts and upper part of the uterus horns were flushed separately with 30ml pre-warmed (38°C) Dulbecco's phosphor buffered saline. Collection media were searched for embryos, which were collected and were stained with Hoechst 33342 for cell count and determination of the development status.

4.2.4 Statistical analysis

Statistical analyses were performed with SIGMA STAT 2.03 for windows (Jandel Scientific Cooperation, San Ragael, CA, USA). The effect of the group treatment on the quality of the semen was analysed for normal distribution and tested 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 $P<0.05$.

4.3 Results

4.3.1 Experiment 1: Improvement of sex sorted fresh boar spermatozoa using Androhep™ supplemented with different antioxidants

Ten ejaculates from four boars were obtained during the experiment. The estimated motility of the samples after collection and dilution with Androhep™ was 74.0±4.36%. The mean concentration of the samples was 280.0±158.03 x 10^6 spermatozoa per ml. The mean percentage of morphological abnormal spermatozoa was 24.93%±12.67%. The percentage of spermatozoa with normal apical ridges was 98.5±0.91%. The percentage of spermatozoa with distal plasma droplets was 20.34±9.45%. The reanalysis of semen revealed 92.5% average purity for X-chromosome bearing spermatozoa.

4.3.1.1 Evaluation of motility after storage and incubation

Table 29 shows the results of the motility evaluation after 0, 24 and 120h of storage at 15°C and subsequent incubation at 38°C. The motility decreased during storage at 15°C as well as during incubation at 38°C in all treatment groups. Sperm motility did not differ between sorted and unsorted spermatozoa except for spermatozoa that were stored for 120h at 15°C and were evaluated after 15min of incubation at 38°C (P≤0.05). The addition of antioxidants had no significant effect on sorted spermatozoa.

Table 29: Sperm motility of fresh and sex-sorted samples after 0, 24 and 120 hours of storage at 15°C and subsequent incubation at 38°C extended in Androhep™ containing different antioxidants or no additives

% motile spermatozoa	**0h storage at 15 °C**		**24h storage at 15 °C**		**120h storage at 15 °C**	
	15min incubation at 38°C x̄±SD	2h incubation at 38°C x̄±SD	15min incubation at 38°C x̄±SD	2h incubation at 38°C x̄±SD	15min incubation at 38°C x̄±SD	2h incubation at 38°C x̄±SD
Unsorted, no additives	70.0±5.9	65.5±6.5	64.4±6.9	55.0±12.9	59.5±5.2[a]	55.0±7.1
Unsorted, + antioxidants	71.0±6.6	66.0±7.0	64.0±6.6	59.0±12.4	60.0±5.5[a]	56.5±5.0
Sex-sorted, no additives	68.0±2.5	54.7±27.0	55.0±13.5	38.3±21.3	22.0±15.8[b]	18.0±15.4
Sex-sorted + , antioxidants	68.3±2.4	60.0±21.5	61.7±6.7	39.4±23.5	28.3±14.7[b]	21.7±19.1

a:b P≤0.05 at same time after thawing between treatment groups

4.3.1.2 Evaluation of sperm morphology after storage and incubation

The evaluation of the percentage of morphological abnormal spermatozoa showed a significantly higher amount of normal apical ridges (NAR) in the unsorted treatment groups compared to sex-sorted samples. Neither for unsorted nor for sex-sorted spermatozoa the addition of antioxidants improved the percentage of spermatozoa with lost acrosomes, except when comparing unsorted spermatozoa in extender with antioxidants vs. sex-sorted spermatozoa without additives ($P \leq 0.05$). The percentage of spermatozoa with dissolving acrosomes was not affected by sorting, although the non-sorted samples had less dissolving acrosomes. However, the difference was only statistically significant when comparing the non-sorted groups with the sex-sorted semen with addition of antioxidants. Table 30 summarizes the results of the morphological analysis.

Table 30: Results of the morphological evaluation of the semen samples after 120h of storage at 15°C

% morphological abnormal spermatozoa after 120h storage at 15°C	normal apical ridge $\bar{x}$±SD	dissolving acrosome $\bar{x}$±SD	lost acrosome $\bar{x}$±SD	other abnormalities $\bar{x}$±SD
Non-sorted, no additives	87.9±4.2[a]	5.2±1.4[a]	6.95±3.4[ac]	23.9±15.0
Non-sorted, + antioxidants	90.4±2.3[a]	4.3±2.0[a]	5.4±3.2[a]	22.3±12.0
Sex-sorted, no additives	63.7±24.3[b]	11.7±9.1[ac]	24.6±16.3[b]	17.3±19.0
Sex-sorted, + antioxidants	62.4±20.6[b]	16.8±8.0[bc]	20.8±15.0[bc]	16.1±22.3

a:b:c $p \leq 0.05$ between treatment groups

4.3.2 Experiment 2: Improvement of sex sorted frozen boar spermatozoa using cooling and freezing media supplemented with different antioxidants

During the experiment six ejaculates were obtained from three boars. The estimated motility of the samples right after collection and dilution with Androhep^{TM} was 75.0±3.16%.
The mean concentration of the fresh samples was 578.3±147.6 x 10^6. The mean percentage of morphological abnormal spermatozoa was 15.2±7.6%. The percentage of spermatozoa with normal apical ridges was 98.8±0.7%.

4.3.2.1 Evaluation of membrane integrity and acrosome reaction

Table 31 displays the results of the flow cytometrical evaluation of membrane integrity and acrosome reaction of the fresh semen samples after collection and 1:2 (v:v) dilution with Androhep™.

Table 31: Results of the flow cytometrical evaluation of membrane integrity and acrosome reaction of **fresh** spermatozoa applying SYTO-17/FITC-PNA/PI staining

Parameter	% spermatozoa $\bar{x}$±SD
vital spermatozoa; intact acrosome	83.9±14.4
vital spermatozoa; acrosome reacted	2.5±1.3
membrane damaged sperm; acrosome non reacted	9.5±11.0
membrane damaged sperm; acrosome reacted	4.0±4.0

4.3.2.2 Evaluation of sperm motility after sorting and freezing

The motility of the spermatozoa after sex sorting and before cooling to 15°C was 65.0±2.8%. Motility after thawing and subsequent maintenance at 38°C (thermo resistance test) was analyzed at 0, 1, 2, 3 and 6h.

Immediately after thawing only the samples from group Sexed-1 (sex-sorted, no additives, 40 x 10^6 sperm per ml; see Table 28) showed a significant lower motility than all other groups ($P \leq 0.05$). After this, no significant differences in the sperm motility could be detected **between** the treatment groups. During the thermo resistance test sperm motility significantly decreased during incubation **within** eight of the ten treatment groups (Table 32), when comparing the percentage of motile spermatozoa 0h vs. 1h, 2h, 3h and 6h after thawing. The treatment groups Sexed-1, Sexed-4 (see Table 28) showed no significant decrase when compared to motility directly after thawing, whereas the treatment-groups Control-4, Sexed-2, -3, ,-5 ,-6 showed a significant decrease in motility after two and the groups Control-2 and Control-3 showed a significant decrease after three hours when compared with the motility directly after thawing. The Control-1 group showed a significant decrease in motility after 6h ($p<0.05$).

Table 32: Percentage of motile spermatozoa after thawing and dilution in AndrohepTM at 0, 1, 2, 3 and 6 hours after thawing

Treatment	0h x̄±SD	1h x̄±SD	2h x̄±SD	3h x̄±SD	6h x̄±SD
Control-1	48.3±17.2^{aA}	43.3±11.7^{A}	25.8±10.7	15.8±3.8	2.8±2.0^{B}
Control-2	55.0±9.5^{aA}	49.2±8.6	30.0±13.8	17.5±11.7^{B}	3.5±3.3^{B}
Control-3	50.8±19.3^{aA}	38.2±18.3^{A}	14.4±6.8^{A}	11.7±6.8^{B}	3.7±2.4^{B}
Control-4	48.0±10.4^{aA}	45.0±8.7^{A}	25.0±10.0^{B}	15.4±7.3^{B}	4.2±4.8^{B}
Sexed-1	16.7±2.6^{b}	13.7±9.5	10.3±9.9	6.7±2.9	1.3±1.9
Sexed-2	23.0±9.7^{aA}	15.0±6.1	10.0±5.0^{B}	10.2±7.6^{B}	3.8±4.3^{B}
Sexed-3	25.8±11.6^{aA}	15.8±5.8	13.3±5.2^{B}	12.5±14.1^{B}	4.3±5.5^{B}
Sexed-4	25.0±14.7^{a}	32.5±17.6	16.3±9.5	13.8±4.8	9.3±7.4
Sexed-5	38.3±8.8^{aA}	30.0±12.6^{AB}	14.8±10.0^{BC}	15.3±11.1^{BC}	4.2±3.1^{C}
Sexed-6	32.5±10.4^{aA}	36.7±11.3^{A}	20.0±5.5^{B}	16.7±9.3^{B}	4.8±3.3^{B}

a:b $p \leq 0.05$ at same time after thawing between treatment groups

A:B:C $p \leq 0.05$ at different time-points within one treatment group

The definition of the treatment groups is displayed in Table 28.

4.3.2.3 Evaluation of sperm morphology after sorting and freezing

Spermatozoa of the different treatment groups were evaluated directly after thawing. Table 33 shows the results of the morphological evaluation. No significant differences were found between treatment groups.

Table 33: Results of the morphological evaluation of the spermatozoa after freezing and thawing

Treatment	% morphologically intact spermatozoa $\bar{x}$±SD	% Normal apical ridge $\bar{x}$±SD	% dissolving acrosome $\bar{x}$±SD	% lost acrosome $\bar{x}$±SD	% other abnormalities $\bar{x}$±SD
Control-1	63.33±11.1	40.0±11.4	3.8±4.8	56.2±9.5	3.3±0.8
Control-2	62.2±11.7	41.2±12.7	6.2±5.8	53.7±12.0	3.3±1.4
Control-3	55.8±7.1	47.3±8.7	3.0±6.0	49.7±8.3	3.2±3.1
Control-4	68.8±11.8	35.2±10.0	2.8±2.8	62.0±7.7	4.0±2.6
Sexed-1	72.3±9.9	29.17±10.9	3.2±3.2	67.7±9.9	1.5±1.9
Sexed-2	63.2±10.2	39.2±11.2	2.4±2.6	58.4±10.1	3.0±2.5
Sexed-3	62.8±15.1	39.5±18.1	3.8±3.7	56.7±16.2	2.3±3.4
Sexed-4	63.8±5.1	38.8±4.3	4.3±5.1	57.0±6.2	2.5±1.0
Sexed-5	65.3±9.4	36.3±10.6	4.2±4.0	59.5±11.0	1.7±1.9
Sexed-6	71.8+8.8	30.5±9.6	3.8±3.1	65.7±8.6	2.3±2.7

no significant differences could be detected

4.3.2.4 Evaluation of membrane integrity and acrosome reaction after thawing

The status of membrane integrity and acrosome reaction were evaluated in the flow cytometer and results were classified in four groups: vital spermatozoa with intact acrosome, vital spermatozoa with reacted acrosome, membrane damaged sperm with non reacted acrosome and membrane damaged sperm with reacted acrosome. Directly after thawing the Control-1 (unsexed, without additives, 40 x 10^6 sperm per ml) spermatozoa showed a significant ($P \leq 0.05$) higher portion of viable sperm with intact acrosomes than the Sexed-4 group (sexed, without additives, 80 x 10^6 sperm per ml) as can be seen in Table 34. Further incubation did not reveal differences among treatment groups . However, in all groups the percentage of vital and acrosome intact spermatozoa decreased significantly ($P \leq 0.05$) during the incubation for six hours (Table 34). Furthermore, the percentage of membrane damaged and acrosome intact spermatozoa was significantly higher ($P \leq 0.05$) after six hours in the groups Control-1, Control-2, Sexed-5 and Sexed-6. The percentage of membrane damaged and acrosome reacted spermatozoa was significantly ($P \leq 0.05$) increased after three hours in the Control-1, Sexed-1, Sexed-2, Sexed-3, Sexed-5 and Sexed-6 and after six hours in the Control-2 group (see Table 28 for definition of groups). No link to either sorting, antioxidants addition or concentration nor sperm concentration could be detected.

Table 34: Results of flow cytometrical evaluation of membrane integrity and acrosome reaction of frozen/thawed spermatozoa applying SYTO-17/FITC-PNA/PI 0h, 3h and 6h after thawing (values are displayed as $\bar{x}$±SD)

Parameter	membrane damaged sperm; acrosome reacted (%)			vital spermatozoa; intact acrosome (%)			vital spermatozoa; acrosome reacted (%)			membrane damaged sperm; acrosome intact (%)		
h after thawing	0h	3h	6h	0h	3h	6h	0h	3h	6h	0h	3h	6h
	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD
Control-1	12.8±3.6^{A}	21.4±6.5^{B}	24.6±5.7^{B}	60.6±5.0^{aA}	42.9±1.8^{B}	35.9±5.3^{C}	1.0±1.3	3.1±0.8	3.1±2.2	25.6±3.6^{A}	32.6±5.8	36.5±9.6^{B}
Control-2	12.9±4.2^{A}	20.0±8.0	23.8±8.1^{B}	58.9±6.4^{A}	44.6±6.2^{BC}	36.6±7.9^{C}	0.8±1.1^{A}	3.2±1.9^{B}	2.2±1.2^{AB}	27.4±3.3^{A}	32.1±5.8	37.4±6.2^{B}
Control-3	13.5±5.8	22.1±9.2	24.3±10.9	57.8±8.3^{A}	40.7±6.2^{A}	35.4±5.7^{BC}	1.1±1.4	2.8±1.1	2.3±1.3	27.7±3.8	34.4±6.1	38.1±9.3
Control-4	15.7±3.6	24.1±8.5	26.5±10.8	56.4±5.5^{A}	40.2±4.6^{BC}	33.0±5.3^{C}	1.1±1.2	2.6±0.4	1.8±0.2	26.8±5.6	33.1±5.8	38.8±8.7
Sexed-1	17.4±1.4^{A}	30.2±2.8^{B}	33.2±5.8^{B}	52.9±5.3^{A}	33.5±4.8^{BC}	26.1±5.4^{C}	1.6±1.4	2.3±1.7	2.0±1.5	28.1±4.687	34.0±7.8	38.7±9.7
Sexed-2	15.4±2.1^{A}	28.3±5.9^{B}	27.8±10.1^{B}	53.2±5.0^{A}	34.6±5.0^{B}	25.9±4.2^{C}	1.6±1.1	2.1±0.7	1.4±0.4	29.8±3.9	35.0±9.3	44.9±12.7
Sexed-3	16.5±4.1^{A}	28.7±7.07^{B}	29.7±8.9^{B}	51.2±7.4^{A}	33.14±3.5	26.5±3.5^{B}	1.4±1.0	2.2±0.43	2.2±1.4	30.8±4.5	35.9±8.4	41.5±11.04
Sexed-4	18.8±4.2	28.8±7.7	26.3±5.5	46.0±7.2^{bA}	31.8±1.9	25.8±1.3^{B}	2.3±1.8	1.8±0.6	1.8±0.8	32.6±7.0	37.4±7.7	46.0±6.0
Sexed-5	15.6±3.1A	28.4±4.4B	27.8±5.6B	51.6±7.9A	31.9±2.2	27.2±3.2B	1.8±1.8	1.9±0.7	1.2±0.5	30.8±7.9A	37.7±5.1	43.6±8.4B
Sexed-6	15.3±2.2^{A}	29.9±4.7^{B}	29.8±5.6^{B}	51.7±4.5^{A}	33.5±3.3^{B}	26.4±3.7^{C}	1.8±1.9	2.2±1.0	1.4±0.6	31.1±5.6^{A}	34.3±5.5	42.1±7.0^{B}

a:b $p \leq 0.05$ at same time after thawing between treatment groups A:B $p \leq 0.05$ at different time-points within one treatment

4.3.3 Experiment 3: Tubal insemination of sex-sorted frozen/thawed boar spermatozoa

The mean motility of the thawed spermatozoa prior to insemination was 39.3±4.7%. From six inseminated gilts 70 embryos were re-flushed. In total 37 (52,5%) embryos were fertilized (based on morphological analysis and all counts). From these 16 embryos (22,9%) remained in early embryo stages (2-8 cells), whereas 19 embryos (51,4% of fertilized embryos or 27,1% of all re-flushed embryos) developed to blastocyst stages and had in average 64.4 cells.

4.4 Discussion

Fresh sex-sorted porcine spermatozoa have successfully been used to produce piglets in swine after artificial insemination with methods applicable on farm (Martinez et al. 2001, Rath et al. 2003a, Grossfeld et al. 2005). Additionally, it is well accepted that cryo-preserved porcine semen allows to produce offspring by normal AI (Johnson 1985) but so far a combination of both methods failed to produce piglets. Only one paper reports a successful surgical insemination of 200.000 frozen/thawed sex-sorted spermatozoa into the oviduct that resulted in the birth of two piglets (Johnson et al. 2000).

In the present study a combination of different antioxidative additives added to semen preservation media was tested for fresh and frozen sexed spermatozoa. Therefore, sperm characteristics were tested and the fertilizing ability of sex sorted spermatozoa was proved by surgical tubal insemination.

The results of this study show that antioxidants added to Androhep™ have no significant effect on the preservation of motility and morphology during liquid storage of boar semen for up to five days. This was true for unsorted as well as sex-sorted spermatozoa. The only difference was detectable after 120 hours of storage between unsorted and sex-sorted semen, where the latter group showed a significantly decreased motility and acrosome morphology. Such effects on sperm quality and fertility have been described previously in several trials with porcine semen as well as in other species (Johnson et al. 2005a, Garner 2006). Reduced fertility results from physical load on sperm during the flow cytometrical separation process were reported from several authors. The effects included pre-capacitation

(Hollinshead et al. 2003, Knöppel 2001, Maxwell et al. 1998), which may be caused by removal of protective substances due to a high dilution during sorting. Motility of spermatozoa was also negatively affected as has been shown in this study and by others (Hollinshead et al. 2003, Rath et al. 2003b). Reasons for a decrease in motility can be related either to the hydro-dynamic differential pressure (Suh and Schenk 2003) or in the electro-static field, necessary to split X- and Y-bearing spermatozoa in two populations. This may cause temporary membrane polarisation of the mid-piece of the sperm tail and disturbs ATP synthesis (Klinc 2005). 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). These findings may explain the lowered motility and membrane status of the sex-sorted sperm samples in this study although the effects were rather low as compared to the unsorted control group.

Reactive oxygen species (ROS) effect sperm integrity negatively and have been found to be build up at several steps during flow cytometrical sorting. These steps include incubation for up to 1.5 hours at 34-37°C (Alvarez and Storey 1985), high dilution with the sheath-fluid (Maxwell et al. 1998), pressure changes (Suh and Schenk 2003) exposure to laser light and centrifugation after sorting (Shekarriz et al. 1995). Especially porcine spermatozoa are prone to peroxidation due to their high content of polyunsaturated fatty acids (Aitken et al. 1989, Cerolini et al. 2000). The negative effects of ROS include inhibition of sperm motility and membrane damages, especially of the acrosome (De Lamirande and Gagnon 1992b, Whittington and Ford 1998).

Although antioxidative additives had been added and should have minimized the effects of ROS, their impact seems to be minimal in comparison to those reported for other species like the bovine (Klinc and Rath 2007, Klinc et al. 2007). As Androhep™ was used in the present experiments for incubation and sorting and ROS may have been already intercepted by the ingredients of the extender that include BSA and a strong buffer system.

If spermatozoa are not only flow cytometrically sorted but additionally frozen and thawed in liquid nitrogen, another source for ROS is added to the process. Cooling

and freezing can cause lipid peroxidation as well (Chatterjee and Gagnon 2001a). It has been shown in the second experiment that sex-sorted spermatozoa, frozen at a concentration of 40 x 10^6 sperm per ml without any antioxidants, had a significantly decreased motility than any other treatment group. Although the effect was not significant, sex-sorted spermatozoa frozen at a concentration of 80 x 10^6 sperm per ml in the presence of antioxidants, showed the highest motility. There were no significant differences in the sperm morphology between the treatment groups, indicating that the sorting process itself prior to freezing is mild enough to allow the sperm membrane system to withstand the freezing forces.

In opposite, if antioxidants were absent, a significant difference was found between unsorted and sorted spermatozoa at a concentration of 40 x 10^6 sperm per ml after freezing and thawing (Sexed-1 vs. Control-1) again indirectly indicating a slight beneficial effect of antioxidants for sorted spermatozoa. This difference could not be detected, when sorted and unsorted samples without additives, but with different sperm concentration (Control-1 vs. Sexed-1 vs. Sexed-4) were compared, indicating a possible concentration effect.

The scavenging effect against ROS were more obvious in other species (Klinc 2005) and with other antioxidants in the porcine. BHT (butylated hydroxytoluene) improved the resistance against cold shock (Bamba and Cran 1992), produced a higher sperm survival after freezing, a lower lipid oxidation and an improved embryo development after IVF (Roca et al. 2004). The addition of Catalase to freezing media for boar semen significantly improved the post thawing quality in boar semen (Roca et al. 2005a) but failed to do so in the equine (Baumber et al. 2005). Also the vitamin E analogue Trolox improved the post thawing quality in boar semen as shown in Chapter I and by Pena et al. (2003b, 2004b). In order to evaluate possible positive effects of these and other antioxidants further research should be directed to further combinations and concentrations of antioxidants.

In order to prove the fertilizing capacity of sex-sorted frozen spermatozoa a surgical insemination trial was performed. In opposite to the experiment by Krueger et al. (1999) semen was not inseminated into the tip of the uterine horn but directly into the ampoule of the oviduct. The idea behind this was to circumvent the utero-tubal junction that is know to have a sperm selective ability (Hunter 1988, Mburu 1997, Suarez et al. 1991, Fazeli et al. 1999).

The earlier experiments by Johnson et al. (1991) had indicated the feasibility to produce offspring by tubal AI when gilts were inseminated with 200.000 sex-sorted frozen spermatozoa and two piglets were obtained. Therefore, six gilts were tubally inseminated with 4 x 10^6 spermatozoa. Altogether 27.1% of all re-flushed embryos developed to blastocyst stages and proved in principle the successful surgical insemination into the oviduct with sex-sorted and frozen/thawed spermatozoa.

It can be concluded from the results of the experiments that sex-sorting and freezing of porcine spermatozoa causes a reasonable stress to the cell organelles. Although reactive oxygen species may be responsible for a larger part of the negative effects other mechanisms of sperm damage may cause significant sperm damages as well. This may be one reason why the antioxidants did not significantly improve the semen quality of porcine spermatozoa after sex-sorting and freezing and thawing. But it allowed the first successful fertilization after surgical tubal insemination.

5. General discussion and conclusions

The results of the experiments in this study have confirmed that freezing and thawing of spermatozoa reduces the sperm motility and increases the percentage of membrane and acrosomal damaged spermatozoa in boar semen. Similar effects have been observed after flow cytometrical sperm sexing. In the presented experiments both insults to spermatozoa have been combined and possible beneficial effects of boar individual freezing speeds and of antioxidant additives in sperm media either during storage in liquid as well as in frozen state were tested.

Improvement of sperm quality after freezing, can be achieved by boar selection, as an animal variety in resistance of spermatozoa to survive freezing exists (Medrano 1998, Holt et al. 2005). If animals are pre-selected for cryo-resistance, the influence of changes in freezing curves is minimized, as has been shown in this study. The sperm quality of only one of four boars showed improvement, when spermatozoa where frozen in three different freezing speeds.

In the presence of Pyruvate, the antioxidants Catalase and Mercaptoethanol had no effect on the sperm quality of semen stored in liquid state. The additives only had a beneficial effect on cryo-preservation in unsorted semen directly after thawing, whereby the presence of Trolox alone did not show a significant positive effect. In contrary to experiments of other research groups, using antioxidants as well (Klinc 2005, Pena et al. 2004b, Cerolini et al. 2001), the additives used in this experiment had no positive effect on the sperm morphological status, nor on the membrane or acrosomal status. This was the case in the experiments with unsexed as well as sexed semen in either fresh or frozen semen. Although boar spermatozoa show a very high content in unsaturated fatty acids in their sperm membranes, the ROS scavenging effects of the used substances were not able to limit damages. Even with doubled concentrations of Pyruvate and the antioxidants Catalase and Mercaptoethanol, no protective effect could be detected in the second experiment of Chapter 2. Further research with different antioxidants and concentrations should evaluate, if possible

negative effects of reactive oxygen species can be prevented during sexing and freezing.

However, other damages than that caused by reactive oxygen species could not be excluded in this study. The laser light involved in the sorting process has been shown in other trials, to cause damages as well. It has been found that sperm motility was inhibited (Montag et al. 2000) and the Ca^{2+} metabolism was negatively affected (Breitbart et al. 1996, Lubart et al. 1997) by exposure to laser light. The pressure changes during sorting and the electro-static field may have a negative influence on spermatozoa as well. Further research in this area is needed as well, to further evaluate the damages to spermatozoa during sorting in order to prevent them.

In conclusion, the antioxidants Catalase and Mercaptoethanol in presence of Pyruvate can have a positive effect on sperm motility after flow cytometrical sorting and freezing directly after thawing. And the additives allowed the first fertilization after surgical tubal insemination in gilts.

6. Summary

Experiments to improve the quality of sex-sorted fresh and frozen porcine spermatozoa

For several reasons the demand for sex sorted porcine spermatozoa has been expressed. This includes advantages for herd management, improved production efficiency, foreseeable restrictions of male castration and gene preservation programmes. Due to the high costs for the sex-sorting process and the reduced storage time in liquid state, freezing of sex-sorted spermatozoa in liquid nitrogen would be very advantageous if the post thawing sperm quality would be acceptable. Therefore, the ultimate goal of the present study was to adapted existing freezing methods for porcine spermatozoa to the post sort processing of sexed spermatozoa.

In two chapters five trials were conducted, in order to improve the quality of frozen- and sex-sorted spermatozoa.
In presence of Sodium Pyruvate, the addition of the ROS scavengers Catalase and Mercaptoethanol, and Vitamin E analogue Trolox to cooling and freezing media of boar semen was tested. Frozen spermatozoa were incubated for up to six hours after thawing and motility, morphological status, membrane integrity, and acrosomal status via triple staining in a flow cytometer (SYTO-17/FITC-PNA/PI) were recorded. In a second trial the influence of individual freezing curves for four boars were tested. Frozen spermatozoa were incubated at 38°C for up to six after thawing and motility as well as morphological status were recorded. Also membrane integrity and acrosome status via triple staining in a flowcytometer (SYTO-17/FITC-PNA/PI) were recorded 0 and 2 hours after thawing.

In the second chapter the addition of Catalase and Mercaptoethanol to media for fresh semen preservation and to cooling and freezing media for cryopreservation were tested. It was also tested if sex-sorted and frozen-thawed porcine spermatozoa can be used for surgical insemination into the tip of the uterine horn. Initially fresh and sex-sorted semen were stored for up to five days with or without the addition of

Pyruvate, Catalase and Mercaptoethanol to the storage media. Semen samples were put in a thermo resistance test at 38°C for two hours after storage at 15°C for 0, 24 and 120 hours and the motility as well as the morphological status after 120 hours were recorded.

In a subsequent trial two different concentrations of Pyruvate, Catalase and Mercaptoethanol were added to cooling and freezing media in two different sperm concentrations (40 and 80 x 10^6 sperm per ml), and motility as well as the morphological status were recorded in a thermo resistance test. Also the membrane integrity and acrosome status were recorded employing a flow cytometrical analysis with a triple staining protocol (SYTO-17/FITC-PNA/PI) 0, 3 and 6 hours after thawing.

Finally, six gilts were inseminated surgically into the ampoule of the oviduct with four million sex-sorted spermatozoa frozen and thawed with addition of Pyruvate, Catalase and Mercaptoethanol. Gilts were slaughtered 48 hours later and the embryos were reflushed from the genital tract.

In summary the following results were obtained.

Directly after thawing fresh spermatozoa frozen in the presence of antioxidants showed a significantly ($P \leq 0.05$) improved motility compared to spermatozoa frozen without additives. The percentage of normal apical ridges and the gross morphology did not differ significantly between the treatment groups. After flow cytometrical analysis of membrane integrity and acrosome status no significant differences could be detected among treatment groups, but the percentage of viable spermatozoa decreased and the percentage of acrosome damaged spermatozoa increased in eleven of twelve sperm subgroups within one respective treatment groups during incubation time.

After the evaluation of different freezing curves for individual animals significant differences could only be detected for one boar directly after thawing. The spermatozoa of this boar showed a higher ($P \leq 0.05$) motility after being frozen with a short, instead with a long freezing curve. The analysis of the morphological status showed no significant differences in all boars between the different freezing curves. The evaluation of the viability and acrosome reaction showed that the percentage of viable, acrosome reacted spermatozoa and the percentage of membrane damaged and

acrosome reacted spermatozoa of one boar were significantly lower in the sample frozen following the medium timed protocol than following the short and long protocol. Significant differences were also recorded in part of the treatment groups of all boars, were the percentage of viable spermatozoa decreased and the percentage of acrosomal damages increased after two hours of incubation at 38°C.

Motility of fresh non-sorted and sex-sorted sperm samples decreased during storage at 15°C as well as during incubation at 38°C in all treatment groups. The sperm motility was higher in the non-sorted groups than in the sex-sorted groups, although the difference was only statistically significant ($P \leq 0.05$) after 120h storage at 15°C and 15min incubation at 38°C. The addition of antioxidants had a slightly beneficial effect on the motility of sex-sorted spermatozoa, but the difference was not statistically significant. The percentage of acrosomal damaged spermatozoa was significantly ($P \leq 0.05$) higher in the sex-sorted spermatozoa than in the fresh semen samples, but did not differ between the samples stored with or without additives.

In the subsequent experiment 0h after thawing only the sex-sorted samples frozen and thawed in sperm concentration of 80 x 10^6/ml without addition of Pyruvate, Catalase and Mercaptoethanol showed a significant ($P \leq 0.05$) lower motility than all other groups. All other groups showed no significant differences. However, the non-sorted semen samples showed a elevated motility when compared with sex-sorted samples, but from 1h after thawing up until 6h no statistically significant differences could be measured in the sperm motility between all treatment groups. No significant differences in the morphological status of the spermatozoa could be detected between all treatment groups. Directly after thawing the non-sorted sperm frozen without antioxidants showed a significantly ($P \leq 0.05$) higher portion of viable sperm with intact acrosomes than the sex-sorted samples frozen without additives and 80 x 10^6 sperm per ml. Further incubation did not reveal significant differences in acrosomes. In all treatment groups the percentage of vital and acrosome intact spermatozoa decreased significantly during incubation for six hours.

The last trial proved that sex-sorted porcine spermatozoa frozen with antioxidants and Pyruvate can successfully be used to fertilize oocytes in vivo after surgical insemination. From the six gilts 70 embryos were reflushed. From these embryos 52.5% were fertilized and 27.1% developed to blastocysts stages.

From these results it can be concluded that the addition of Pyruvate, and the antioxidants Catalase and Mercaptoethanol have only limited effect on motility of fresh, sex-sorted and frozen porcine spermatozoa and almost no effect on sperm morphology. Antioxidants did not significantly improve the quality of sex-sorted semen after either fresh or frozen storage. There may be other damages during flowcytometrical sorting that can not be prevented by the addition of the ROS scavengers.

7. Zusammenfassung

Experimente zur Verbesserung der Qualität von gesextem und tiefgefrorenem Ebersperma

Aus verschiedenen Gründen gibt es einen Bedarf für gesextes Ebersperma. Einige dieser Gründe sind Vorteile im Herdenmanagement, verbesserte Produktionseffizienz, Restriktionen bei der Kastration männlicher Tiere und Programme zur Genpräservation. Aufgrund der hohen Kosten für den Sortierprozess und der geringen Lagerdauer in Flüssig konserviertem Zustand, würde das Tiefgefrieren von gesextem Sperma große Vorteile bringen, wenn die Spermaqualität nach dem Auftauen akzeptabel wäre. Daher war das Ziel dieser Studie existierende Tiefgefriermethoden für Schweinesperma an die Verarbeitung von flowzytometrisch gesextem Sperma anzupassen.

In zwei Abschnitten wurden fünf Versuche durchgeführt, um die Qualität von tiefgefrorenem und gesextem Sperma zu verbessern.
Im **ersten Abschnitt** wurde zunächst der Zusatz von Na-Pyruvat, Catalase und Mercaptoethanol und des Vitamin E-Analogs Trolox zum Kühl- und Gefrierverdünner von Ebersperma getestet. Die tiefgefrorenen und aufgetauten Spermien wurden bis zu sechs Stunden nach dem Auftauen inkubiert und die Motilität, der morphologische Status und die Membranintegrität und der Akrosomstatus wurden mittels Färbung mit SYTO-17/FITC-PNA/PI im Flowzytometer getestet. In einem zweiten Versuch wurde der Einfluss von individuellen Einfrierkurven für vier Eber getestet. Die tiefgefrorenen und aufgetauten Spermien wurden bei 38°C für bis zu sechs Stunden nach dem Auftauen inkubiert und die Motiliät, der morphologische Status wurde ebenso erfasst. Ebenso wurde die Membranintegrität und der Akrosomstatus mittels Färbung mit SYTO-17/FITC-PNA/PI im Flowzytometer getestet.

Im **zweiten Abschnitt** wurde der Zusatz von Na-Pyruvat, Catalase und Mercaptoethanol zu flüssig konserviertem frischem und gesextem Sperma und zum

Kühl- und Gefrierverdünner für die Spermatiefgefrierung getestet. Ebenso wurde getestet, ob gesexte und tiefgefrorene Eberspermien für die chirurgische Besamung in die Uterushornspitze genutzt werden können. Zunächst wurden frische oder gesexte Eberspermien bis zu fünf Tage mit oder ohne Zusatz von Pyruvat, Catalase und Mercaptoethanol zum Konservierungsmedium gelagert. Nach Lagerung für 0 Std., 24 Std. oder 120 Std. wurden Proben entnommen und für bis zu zwei Stunden bei 38°C gelagert. Die Motilität und der morphologische Status nach 120 Std. wurden erfasst.

In einem folgenden Versuch wurden zwei verschiedenen Konzentrationen der Antioxidantien zum Kühl- und Gefrierverdünner und zwei verschiedene Spermakonzentrationen von ungesextem und gesextem Ebersperma getestet. Die Motilität im Thermoresistenztest und der morphologische Status wurden erfasst. Ebenso wurden die Membranintegrität und der Akrosomstatus mittels Färbung mit SYTO-17/FITC-PNA/PI im Flowzytometer nach 0 Std., 3 Std. und 6 Std. nach dem Auftauen getestet. Zuletzt wurden sechs Jungsauen chirurgisch mit vier Millionen gesexten und unter Zusatz von Antioxidantien tiefgefrorenen Eberspermien in die Eileiterampulle besamt. Die Jungsauen wurden 48 Std. später geschlachtet und die Embryonen wurden aus dem Genitaltrakt zurückgespült.

Die folgenden Ergebnisse lassen sich zusammenfassen:

Im **ersten Abschnitt** zeigte das Vorhandensein von Na-Pyruvat, Catalase und Mercaptoethanol zum Kühl- und Gefrierverdünner eine signifikant ($P \leq 0,05$) verbesserte Spermienmotilität direkt nach dem Auftauen verglichen mit Sperma, das ohne jegliche Zusätze eingefroren wurde. Der Prozentsatz von Spermien mit normalem apikalem Rand und gesamter morphologisch veränderter Spermien unterschied sich nicht zwischen Gruppen. Ebenso zeigte die flowzytometrische Analyse der Membranintegrität und des Akrosomstatus keine signifikanten Unterschiede. Allerdings sank der Prozentsatz von lebenden Spermien und stieg der Prozentanteil von Akrosom reagierten Spermien signifikant ($P \leq 0,05$) innerhalb von elf von 12 Behandlungsgruppen im Zeitverlauf an.

Nach der Analyse von drei verschiedenen Einfrierkurven für die vier Eber konnten signifikante ($P \leq 0,05$) Unterschiede zwischen den Einfrierkurven nur bei einem Eber festgestellt werden. Die Spermien dieses Ebers zeigten eine höhere Motilität nach

dem Auftauen, nachdem sie mit einer kurzen, anstatt einer langen Einfrierkurve tiefgefroren wurden. Die Auswertung des morphologischen Status der Spermien zeigte keine signifikanten Unterschiede zwischen den jeweiligen Einfrierkurven. Die Analyse der Spermien im Flowzytometer zeigten einen niedrigeren Anteil viabiler und Akrosom reagierter Spermien und einen signifikant ($P \leq 0,05$) niedrigeren Anteil Membran geschädigter und Akrosom reagierter Spermien bei einem Eber dessen Spermien mit einer mittleren Einfrierkurve, anstatt der kurzen oder langen Kurve eingefroren wurden. Signifikante ($P \leq 0,05$) Unterschiede wurden in Teilen der Versuchsgruppen bei allen Ebern gefunden, wo der Prozentanteil viabiler Spermien absank und der Prozentanteil Akrosom reagierter Spermien anstieg, jeweils nach zweistündiger Inkubation bei 38°C.

Im zweiten Abschnitt sank die Motilität von frischen ungesexten und gesexten Eberspermien während der Lagerung bei 15°C und bei der Inkubation bei 38°C in allen Behandlungsgruppen ab. Die Spermamotilität war höher in den ungesexten, als in den gesexten Versuchsgruppen, allerdings war dieser Unterschied nur statistisch signifikant ($P \leq 0,05$) nach einer Lagerdauer von 120 Std. bei 15°C und 15 Minuten Inkubation bei 38°C. Der Zusatz von Pyruvat, Catalase und Mercaptoethanol zeigte einen leichten positiven Effekt auf die Motilität bei den gesexten Spermien, aber die Differenz war nicht signifikant. Der Anteil von Akrosom geschädigten Spermien nach morphologischer Analyse war in den gesexten Versuchsgruppen signifikant ($P \leq 0,05$) höher als in den ungesexten Versuchsgruppen, unterschied sich aber nicht zwischen Gruppen mit oder ohne Zusatz von Pyruvat, Catalase und Mercaptoethanol. Im folgenden Experiment direkt nach dem Auftauen zeigten nur gesexte Spermien, die bei einer Spermakonzentration von 80 Mio. Spermien pro ml ohne Zusätze eingefroren wurden, eine signifikant ($P \leq 0,05$) niedrigere Motilität als alle anderen Versuchsgruppen. Zwischen allen anderen Gruppen bestanden keine signifikanten Unterschiede. Die ungesexten Versuchsgruppen zeigten eine höhere Motilität als die gesexten Versuchsgruppen. Dieser Unterschied war aber von einer Stunde bis sechs Stunden nach dem Auftauen nicht signifikant. Bei der morphologischen Beurteilung der Spermien konnten ebenfalls keine signifikanten Unterschiede festgestellt werden. Direkt nach dem Auftauen zeigte die Gruppe unsortierter, mit einer Konzentration von 40 Mio. Spermien pro ml und ohne Zusätze eingefrorene Gruppe einen

signifikant (P≤0,05) höheren Prozentsatz von viabilen Spermien mit intakten Akrosomen als gesexte Spermien, mit Zusatz von Pyruvat, Catalase und Mercaptoethanol, die bei einer Konzentration von 80 Mio. Spermien pro ml eingefroren wurden. Nach einer Inkubation von einer Stunde und mehr konnten keine signifikanten Unterschiede mehr festgestellt werden. In allen Versuchsgruppen sank der Anteil viabiler und Akrosom intakter Spermien signifikant (P≤0,05) während der Inkubation ab.
Im letzten Versuch konnte nachgewiesen werden, dass gesexte Eberspermien, die mittels Zusatz von Pyruvat, Catalase und Mercaptoethanol tiefgefroren wurden erfolgreich Oozyten nach chirurgischer Besamung in vivo befruchten können. Von sechs Jungsauen konnten 70 Embryonen zurückgespült werden. Von diesen Embryonen waren 52,5% befruchtet und 27,1% entwickelten sich zu Blastozysten-Stadien.

Zusammengefasst hat der Zusatz von Pyruvat und der Antioxidantien Catalase und Mercaptoethanol zu Verdünnermedien nur einen geringen Effekt auf frische, gesexte oder tiefgefrorenen Eberspermien. Der Zusatz kann die Motilität der Spermien verbessern, hat aber nur einen geringen oder keinen Effekt auf die Spermienmorphologie. Der Zusatz von Pyruvat, Catalase und Mercaptoethanol verbesserte die Qualität von gesextem und flüssig konserviertem oder tiefgefrorenem Sperma nicht signifikant. Möglicherweise gibt es andere Schädigungen beim Spermasexing, die nicht durch den Zusatz von Pyruvat, Catalase und Mercaptoethanol verhindert werden können.

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9. List of Tables

10. List of Figures

DECLARATIONS

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

   Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

   Göttingen, 26. May 2007

   …….
   Rudolf Großfeld

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

   Göttingen, 26. May 2007

   …….
   Rudolf Großfeld

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