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Studies on the interaction of chromatin-unstable boar sperm with the female reproductive tract

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To
Rodney

and
my parents
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List of Abbreviations

°C Degrees, Celcius
µg microgram (s)
µl microliter (s)
AI Artificial Insemination
ART Assisted Reproductive Techniques
AO Acridine Orange
AOT Acridine Orange Test
BI Binding Index
BSA Bovine Serum Albumin
BTS Beltsville Thawing Solution
DMSO Dimethylsulfoxde Anhydrous
DNA Deoxyribonucleic acid
DTT 1,4-dithiothreitol
EDTA Ethylenedinitrilo Tetraacetic Acid
et al. et alii (and others)
g Gram (s)
h hours
HGM Hannover Gilt Model
ICSI Intracytoplasmatic Sperm Injection
IVF In Vitro Fertilization
L liter (s)
mfSCSA modified fluorescent microscopic Sperm Chromatin Structure Assay
mHz megahertz
min minute (s)
ml mililiter (s)
mOsm/kg miliosmol per kilogram
n number
OEA Porcine Oviduct Explant Assay
P Probability; level of statistical significance
PBS Phosphate Buffered Solution
pH  negative logarithm of the effective hydrogen-ion concentration in gram equivalents per liter of a solution, used to measure the degree of alkalinity or acidity in a solution.

PI  Propidium Iodide

PMN  Polymorphonuclear leukocytes

R  correlation coefficient

ROS  Reactive Oxygen Species

SAS  Statistical Analysis Systems

SCSA  Sperm Chromatin Structure Assay

SD  Standard deviation

TALP  Tyrode-Albumin-Lactate-Pyruvate

UTJ  utero-tubal junction

vs.  versus
1 Introduction

The goal of semen quality evaluation is the assessment of the ability of sperm to establish pregnancy successfully. This ability includes the capacities to transverse and to survive in the female reproductive tract; to reach, penetrate and fertilize the oocyte; and to support embryo development. To evaluate how and to what extent a given defect could affect fertility, it has to be determined if said defect prevents the sperm from coming into contact with the oocyte or if it hinders embryonic development.

Sperm defects traditionally evaluated include traits relatively easy to assess, such as motility or morphological abnormalities. Access to new technologies has refined semen evaluation, making it possible for researchers to investigate different, complex sperm functions and morphological characteristics. As a result, the list of sperm defects screened has grown considerably and includes physiological aspects that were either unknown or whose evaluation was not feasible with the technology available in the past. It is expected that this trend will continue.

Of the millions of inseminated sperm, very few will interact with the oocyte and be able to penetrate the zona pellucida. This reduction is caused by strict mechanisms of sperm selection in the female reproductive tract that ensure that only sperm with high fertilization potential will be able to reach and penetrate the oocyte (HARRISON, 2000; GUALTIERI and TALEVI, 2003; HOLT and VAN LOOK, 2004). Defects that are detected by the reproductive tract, causing the defective sperm to be culled, therefore unable to reach the oocyte, are called “compensable defects”. This term indicates that using a higher number of spermatozoa per dose can reduce the detrimental effect of these sperm defects on fertility (SAACKE, 2000; AMANN and HAMMERSTEDT, 2002). Other sperm, however, appear to be normal in their motility and morphology; therefore, they will not be rejected by the reproductive tract. They are able to initiate fertilization, but have deficiencies that prevent either the fulfillment of fertilization or a normal embryo development (SETCHELL et al., 1988; BARTH, 1992; DEJARNETTE et al., 1992). These types of deficiencies are called “uncompensable defects” and affect the reproductive parameters, regardless of how many spermatozoa are present in the insemination dose (SAACKE et al., 1998; SAACKE et al., 2000; EVENSON et al., 2000).
A sperm defect that has been the focus of recent studies is chromatin structure instability. Sperm chromatin structure is very compact and stable, thus quite able to protect its genetic integrity during the sperm transport through the male and female reproductive tracts (EVENSON et al., 2002). A defective chromatin condensation renders the chromatin unstable and sensitive to denaturing stress (AHMADI and NG, 1999). It is believed that chromatin instability leads to poor fertilization or embryo development (bull: BALLACHEY et al., 1987; JANUSKAUSKAS et al., 2003; KHALIL, 2004. Mice: AHMADI and NG, 1999. Human: LIU and BAKER, 1992; SPANO et al., 2000; BUNGUM et al., 2004).

It is generally assumed that the reproductive tract does not select sperm based on the stability of its chromatin, i.e., that sperm instability is an uncompensable defect. Study has recently begun on chromatin instability and little data is available regarding the prevalence of the defect or its effect on fertility in the pig. Information from other species shows no constant correlation between chromatin abnormality and other sperm defects (Bull: JANUSKAUSKAS et al., 2003; KHALIL, 2004. Human: IBRAHIM and PEDERSEN 1988; LIU and BAKER, 1992; MOLINA et al., 2001; FISCHER et al., 2003). In a recent study on porcine sperm (WABERSKI et al., 2002), no correlation between chromatin instability and other sperm parameters was found.

The aim of this study was to elucidate the relevance of sperm chromatin instability for the fertilization capacity of boar semen. This was achieved using in vitro and in vivo models to analyze the interaction of chromatin unstable sperm with the female reproductive tract. It was investigated whether, and to what extent, sperm with unstable chromatin has the capacity to bind to the oviductal epithelia in vitro, which is a step required to establish the sperm reservoir. The prevalence of chromatin instability in the fertilizing-competent sperm population in the oviduct in vivo was studied through qualitative analysis of the chromatin status of accessory sperm found in embryos. Additionally, the use of quantitative analysis of accessory spermatozoa to study the effect of chromatin disturbances and other sperm defects on fertilization capacity is shown.
2 Review of Literature

2.1 Sperm selection in the female reproductive tract

Sperm selection is a mechanism that ensures that only sperm that can successfully participate in fertilization will reach the fertilization site and be able to penetrate the zona pellucida (HARRISON, 2000; GUALTIERI and TALEVI, 2003; HOLT and VAN LOOK, 2004). This mechanism is necessary, because the quality of ejaculated sperm in mammals is morphologically and physiologically heterogeneous (HARRISON, 2000). Another reason for the evolution of the selection mechanism is the prevention of polyspermy, i.e. the penetration of the oocyte by more than one sperm. A high degree of polyspermy was found in experiments where the number of sperm at the site of fertilization was increased (DAY and POLGE 1968; HUNTER 1972; HUNTER and NICHOL, 1988). Thus, the selection process is also associated with a severe reduction in the number of sperm, from the millions ejaculated to the few present at the site of fertilization near the time of ovulation (GUALTIERI and TALEVI, 2003). The selection process in the pig takes place through the passage of the utero-tubal junction, in the oviduct and at the zona pellucida of the oocyte. A considerable amount of sperm is lost in the uterus, but these losses have not yet been correlated to specific defects.

The spermatozoa losses and selection translate into a gradient in the number of sperm cells found in different parts of the female reproductive tract (Figure 1), which goes from $10^9$ cells in the artificial insemination (AI) dose (JOHNSON et al., 2000) to $10^8$ on the uterine side of the utero-tubal junction (HUNTER, 1988) and $10^5$ at the uterine end of the oviducts (VIRING and EINARSSON, 1980). VIRING and EINARSSON (1980) divided the isthmus into four segments and found a gradient in the spermatozoa number, from $10^5$ at the uterine end of the oviducts, to $10^3$ at the beginning of the second half of the oviducts. By the ampullary end of the isthmus, this number decreased to $10^2$ cells (VIRING and EINARSSON, 1980; MBURU et al., 1996).
2.1.1 Criteria and location of selection

_Uterus_. In the uterus, two different kinds of sperm losses occur: backflow, where sperm are expelled from the uterus via the vagina, and phagocytosis of the sperm cells, done by
polymorphonuclear leukocytes (PMN). No specific mechanisms of selection have been
determined to date; both appear to be random processes.

Backflow takes place during or shortly after insemination (STEVERINK et al., 1988). Several
studies have agreed on backflow as a major cause of spermatozoa loss. Up to 45% of the
inseminated sperm will be lost in this manner in the first hour after insemination (VIRING
and EINARSSON, 1981; STEVERINK et al., 1998; MATTHIJS et al., 2000a). The quantity
of sperm lost by backflow seems to depend on the volume of the insemination dose
(MATTHIJS et al., 2003) and varies considerably among sows (STEVERINK et al., 1998).
Backflow is believed to be a normal physiological phenomenon: it is present in every sow
and, apparently, it does not have a detrimental effect on fertility, as long as the number of
sperm inseminated is not too low (STEVERINK et al., 1998; ROZEBOOM et al., 2000).
Backflow is not related to timing of insemination; therefore, the presence of backflow does
not indicate incorrect timing of insemination in relation to ovulation (STEVERINK et al.,
1998). It is possible that backflow is related to the size of the uterus: BAKER and DEGEN
(1972) collected a larger volume of backflow from first parity sows (which have a smaller
uterus) than from multiparous sows.

The presence of seminal plasma and spermatozoa produces an inflammatory reaction in the
sow. About 30 min after insemination, PMN and macrophages are present in the uterus and
attack the sperm cells (LOVELL and GETTY, 1968; ROZEBOOM et al., 2000; MATTHIJS
et al., 2000, 2000a). Phagocytosis has the function of removing excess sperm and bacteria
present in the ejaculate from the uterus (ROZEBOOM et al., 2000). Through this clearance,
the uterus will be ready to receive the embryos that could arrive as soon as 48 h after
ovulation (ROZEBOOM et al., 2000; WOELDERS and MATTHIJS, 2001; RODRIGUEZ-
MARTINEZ et al., 2005). The magnitude of the immune response seems to depend on the
number of spermatozoa inseminated, although the reaction itself is triggered by the infusion
of any liquid solution (MATTHIJS et al., 2000, 2003). PMN are found in the uterine lumen 12
h after insemination, however, only when the solution infused contains sperm (WOELDERS
and MATTHIJS, 2001). The number of PMN recruited to the uterine lumen increases rapidly
at the beginning of the inflammatory reaction; by 4 h after insemination, the number of PMN
is approximately 30 times that of non-phagocytosed spermatozoa (MATTHIJS et al., 2000).
The number of PMN reaches a plateau between 6 and 12 h after insemination (ROZEBOOM
et al., 1998) and then decreases gradually: at 24 h the number is significantly reduced in
comparison to those found 2-8 h after insemination (PURSEL et al., 1978), at 27 h only degenerating leukocytes are observable (LOVELL and GETTY, 1968), and at 35 h PMN finally disappear from the uterine lumen (ROZEBOOM et al., 2000). WOELDERS and MATTHIJS (2001) found no evidence that damaged, aging or dead sperm would be deliberately targeted for phagocytosis. Phagocytosis affects only sperm present in the uterus, as no leukocytes are present in the oviducts (HUNTER, 1997).

**Utero-tubal junction.** The utero-tubal junction (UTJ) presents a barrier for sperm, through which mainly those not hindered by certain defects can pass. Thus, sperm that are dead (OVERSTREET and COOPER, 1978), immobile (HUNTER, 1975; OVERSTREET and COOPER, 1978; MULLINS and SAACKE, 1989) or have abnormal tail movements (HOLT and VAN LOOK, 2004), as well as those with severely abnormal head morphology (SAACKE et al., 2000), will be less likely to access the oviducts than sperm without these defects (SAACKE et al., 2000).

**Oviduct.** Sperm bind to the epithelia of the lower oviductal isthmus, thereby forming the sperm reservoir, which has several functions, including sperm selection. In vitro studies have provided growing evidence that the binding of the sperm to the oviductal epithelia, per se, represents a selection mechanism (WABERSKI et al., 2005). In order to bind to the oviduct, sperm must fulfill certain morphological and physiological requirements. Studies in vitro have shown that the population of sperm bound to the oviductal epithelia has higher percentages of motility (SUAREZ et al., 1991; THOMAS et al., 1994; ELLINGTON et al., 1999), normal morphology (SUAREZ et al., 1991), viability and intact plasma membrane (ELLINGTON et al., 1999; PETRUNKINA et al., 2001), as well as a lower Ca²⁺ concentration (PETRUNKINA et al., 2001), than sperm in the ejaculate. It was also observed that sperm bound to the oviductal epithelia in vitro showed a suppressed tyrosine phosphorylation (PETRUNKINA et al., 2001) and that only sperm with intact acrosomes bound to the oviducts in vitro (GUALTIERI and TALEVI, 2000). Additionally, GUALTIERI and TALEVI (2003) observed in vitro that bull spermatozoa that were able to bind to the oviduct showed a higher zona pellucida binding and fertilization competence than those which could not bind. Studies in vivo, on the other hand, showed that capacitated or hypermotile sperm were not able to bind to the oviduct (SMITH and YANAGIMACHI, 1991) and that oviduct bound sperm possess an intact sperm plasma membrane and acrosomes (MBURU et al., 1997). Sperm that reach the
oviducts, but that are not able to bind to the epithelia, traverse the oviducts and the infundibulum and are lost in the peritoneal cavity (DEGEN and HAWES, 1972).

Zona pellucida. The sperm needs to undergo “capacitation” in order to be able to penetrate the zona pellucida. Capacitation has been defined as “a set of changes in the sperm plasma membrane that enables sperm to undergo the acrosome reaction” (SUAREZ, 2001). Capacitated sperm cells can recognize and bind to the zona pellucida of the oocyte and, upon binding, undergo the acrosome reaction. The acrosome reaction involves the release of certain enzymes that facilitate the passage of the sperm cell through the zona (HARRISON, 1996). The sperm not only need to be capacitated to penetrate the zona pellucida, but also need to be vigorously motile, or even hypermotile (YANAGIMACHI, 1994; OLDS-CLARKE, 1996; HARRISON, 1996). Motility hyperactivation involves a significant increase in flagellar bend amplitude and, usually, in beat asymmetry, as well (SUAREZ, 2001). The wider amplitude and the asymmetry of the movement are believed to facilitate the encounter with the oocyte (STOREY, 1995). It is important to note that the population of sperm that reaches the zona pellucida in vivo has been subjected to strict selection by the previous “checkpoints” of the reproductive tract (i.e. uterus, UTJ, oviducts). This evidently diminishes the need for a major selection process specific to the zona pellucida. The increasing use of assisted reproductive techniques (ART), especially in vitro fertilization (IVF), has encouraged the study of the selection of sperm at the zona pellucida in vitro. Sperm bound to the zona pellucida after IVF treatment showed higher percentages of normal morphology (LIU and BAKER, 1992a). It has been observed that acrosome-reacted sperm (LIU and BAKER, 1992a) and sperm with abnormal acrosome morphology (THUNDATHIL et al., 2000, 2001) had little or no ability to bind to the zona. Additionally, sperm with severely abnormal head morphology were not able to completely penetrate the zona pellucida in vitro (HOWARD et al., 1993).

2.1.2 Compensable sperm defects

Seminal deficiencies that prevent the sperm from coming into contact with or penetrating the oocyte are called “compensable defects” (SAACKE et al., 2000). The use of a higher number of spermatozoa per dose can reduce the negative effect that this type of defect has on fertility (AMANN and HAMMERSTEDT, 2002). This might explain why fertility increases with increasing numbers of sperm inseminated up to a threshold, after which limiting factors in the reproductive capacity of the female become important (Figure 2) (SALISBURY and VAN
DEMARK, 1961; DEN DAAS, 1992). Such a relationship exists for sperm defects like progressive motility, acrosomal integrity and cell membrane integrity (PACE et al., 1981), as well as for severe sperm head and tail morphological abnormalities, which hinder the migration of the sperm through the narrow folds of the UTJ (SAACKE et al., 2000). However, there are compensable sperm defects that cannot be evaluated with the conventional assessments of sperm viability or morphology. These defects would include molecular events on the sperm surface or functional changes of sperm associated with ability to colonize the sperm reservoir, as well as the ability to undergo capacitation, to recognize the oocyte and to undergo the acrosome reaction, none of which have been accurately evaluated under standardized laboratory conditions (SAACKE, 2004).

**Figure 2. Relation between non-return rate and total number of spermatozoa inseminated.** The sperm from different bulls differ in the maximum non-return rate and in the rate at which the asymptote is approached. There is no correlation between the asymptotic non-return value and the rate at which this value is approached. This implies that the bull ranking will change with the total number of spermatozoa in the insemination dose as long as this dose remains below the threshold of optimal fertility (Modified from DEN DAAS, 1992).
2.1.3 Uncompensable sperm defects

Some apparently normal sperm possess deficiencies that allow the sperm to fertilize, but prevent the fulfillment of fertilization or a normal embryo development (SETCHELL et al., 1998; BARTH, 1992; DEJARNETTE et al., 1992). These types of deficiencies are called “uncompensable defects” (SAACKE et al., 2000). They will affect the reproductive parameters, regardless of how many spermatozoa are present in the insemination dose (SAACKE et al., 1998; EVENSON et al., 2000). Some uncompensable defects have been described through the study of the accessory sperm (SAACKE et al., 2000). Accessory sperm are those that are able to reach the oocyte and partially penetrate the zona pellucida (DEJARNETTE et al., 1992) and are believed to be capable of fertilization (SAACKE et al., 2000). Thus, defects found in accessory sperm in the same percentage as in the inseminated sperm would be classified as uncompensable. For example, minor head morphological abnormalities are regarded as uncompensable (SAACKE et al., 2000). Another defect typically considered within this category is a defective chromatin structure, (GLEDHILL, 1970; SAKKAS et al., 1999; TOMLINSON et al., 2001), which will be described below (see 2.3).

2.1.4 In vitro models used to study sperm-oviduct interaction

The binding of sperm to the oviduct appears to be a selection mechanism, as mentioned above. Because of this, sperm quality and selection criteria have been studied by measuring the capacity of sperm to bind to the oviductal epithelia in vitro. Both the characteristics and the number of oviduct bound sperm can be determined. Although in vitro systems lack the effect that the local signal transduction between oviduct and surrounding tissues and organs as well as the physical properties of oviductal secretions (HUNTER et al., 1998), they allow cellular and molecular studies of basic principles of sperm-oviduct interaction (PETRUNKINA et al., 2001). In most systems dealing with bovine oviducts, the epithelia is squeezed out of, or scraped from, the oviduct (DE PAUW et al., 2002; GUALTIERI and TALEVI, 2003). Although this same approach has been used in some studies in the pig (GREEN et al., 2001), in other studies small pieces of the epithelia are cut from the longitudinal folds of open oviducts.
Oviductal Monolayers. Oviductal monolayers are epithelial cells recovered from oviducts (ampulla and isthmus) and then cultured for up to 10 days, when cell confluence occurs. In the model presented by GUALTIERI and TALEVI (2003) the monolayers are commonly used within 48 h after confluence. Bull semen is first subjected to Percoll gradient centrifugation and then coincubated with the oviductal monolayers in a 5% CO\textsuperscript{2} atmosphere at 38.5°C for one hour. The number of oviduct bound sperm is determined by analyzing 10 fields per sample. Oviduct monolayers have been used in a number of studies in different animal species, e.g. cattle: DE PAUW et al., 2002 and pigs: RAYCHOU DHURY and SUAREZ, 1991; GREEN et al., 2001. In addition to being used to estimate sperm binding capacity, oviductal monolayers can be employed for time-course studies with prolonged co-incubation time of sperm and oviductal cells (POLLARD et al., 1991). Advantages of this method include greater cell homogeneity than in non-cultured tissue explants and the possibility both to store cultured cells in frozen status and to produce permanent cell lines (BOULLANT et al., 1975). However, cultures can be affected by cell dedifferentiation, loss of cell polarity and changes in cell morphology, which could hinder the evaluation of important binding mechanisms and decrease the binding ability of the sperm (FRESHNEY, 1992; BAILLIE et al., 1997).

Porcine Oviduct Explant Assay (OEA). This \textit{in vitro} model evaluates the semen based on its ability to bind to small oviduct pieces, which are called explants (PETRUNKINA et al., 2001). The OEA is based on the assay developed by SUAREZ et al. (1991). Briefly, the oviductal explants (0.5-1 mm) are cut from the longitudinal folds of the isthmic part of oviducts collected from multiparous sows after slaughter. Explants are selected based on the ciliary movement observed. Semen is first subjected to Percoll gradient centrifugation and then coincubated with two explants. The coincubation takes place under a 5% CO\textsuperscript{2} atmosphere at 38.5°C and lasts 15 min. The semen evaluation done using the “Binding Index” (BI), i.e. number of sperm bound per 0.01 mm\textsuperscript{2} of epithelia. The BI is calculated by adding up the number of sperm bound to each of three videotaped sections of an explant and dividing it by the sum of the surface area of the same three sections for two different explants, before determining the arithmetic mean of the two explants (see 3.3.1.6). SIDHU et al. (1999) have used a similar approach to study sperm-oviduct interaction in the brushtail possum. The binding index does not appear to be influenced by the region of the oviduct used (ampulla vs. isthmus), the parity of the female (gilts vs. sows) or day of estrus cycle (day 0 vs. 10) (SUAREZ et al., 1991; PETRUNKINA et al., 2001). A study in humans has reported that
more sperm bound to isthmic than to ampullary explants and that more sperm are able to bind to explants than to cultured monolayers (BAILLIE et al., 1997). Although the life span of the explants is limited to a few hours after collection, they preserve most properties of the original epithelium, most importantly its morphological differentiation and ciliary activity. Thus, this model might be closer to the situation in vivo than the cultured monolayers method (PETRUNKINA et al., 2001).

### 2.1.5 In vivo models used to study sperm fertilizing capacity

The fertilization conditions in vitro are clearly different from those in vivo (HARRISON, 1997). Therefore, in vivo models are needed to test improvements in the fertilizing capacity of sperm, before a new product reaches the market. Assessment of fertilizing capacity of semen in vivo presents a challenge because it is influenced not only by semen-related factors but also by female fertility and by many other sources of variation, which may or may not be determinable (AMANN and HAMMERSTEDT, 2002). It is often the case in field trials that high numbers of sperm are inseminated and that the data regarding pregnancy rate and litter sizes is questionable; therefore, a large number of females is required to minimize the effect of the different variation sources. In contrast to field trials, the in vivo models presented here do not evaluate semen quality based on farrowing rates and litter size, but are based on the fertilization rate, the rate of normal embryos and the accessory sperm count in day 3-5 embryos. Alternatively, in vivo models might use the number of normally developed day 30 fetuses instead of the fertilization rate of day 3-5 embryos; this approach offers a more reliable prognosis of farrowing rates and litters sizes (WABERSKI et al., 1994). When applied under standardized conditions, in vivo insemination models are more sensitive detectors of fertilization differences than field trials, because many of the sources of variation can be eliminated. Additionally, fewer animals are needed and the trials can be performed in shorter periods of time than in field trials (ARDON et al., 2003).

The Hannover Gilt Model (HGM) was established at the School of Veterinary Medicine Hannover (WEITZE et al., 1990). Under this model, rather than employing hormones to induce ovulation, spontaneously ovulating gilts are used to preserve the physiological conditions of fertilization (i.e. ovulation rate, sperm transport and oviductal environment). These gilts are inseminated just once, thus allowing for the determination of the insemination to ovulation interval i.e. sperm aging in vivo, which can influence the fertilization results
The time of ovulation is detected through transcutaneous ultrasound examination of the ovaries every 12 h. The semen doses contain low numbers of sperm (e.g., \(0.8 \times 10^9\) sperm/dose), so that differences in semen quality can be measured accurately; higher sperm doses (e.g., commercial sperm doses of \(2-3 \times 10^9\) sperm cells) can mask the effect of compensable defects (SAACKE et al., 2000; AMANN and HAMMERSTEDT, 2002). Day 3-5 embryos are recovered post-slaughter for evaluation. SOEDE et al. (1995) and KEMP et al. (1996) use a similar model. This model differs from the HGM in that multiparous sows are used instead of gilts. MBURU et al. (1998) have also used a similar model.

2.2 Sperm reservoir

The interval between the beginning of estrus and ovulation in the pig varies and can be very long in certain sows (up to 120 h); therefore, ovulation cannot be predicted based on the onset of estrus (WEITZE et al., 1994; SOEDE et al., 1995). When insemination takes place before ovulation, viable and competent sperm cells are stored in the “functional sperm reservoir” while awaiting ovulation. It has been suggested that viable sperm could be stored as long as 36 h, if not longer (HUNTER, 1997). This does not mean, however, that the quantity of viable stored sperm will be enough to obtain satisfactory fertilization rates. Although fertilization has been observed in sows inseminated as much as 48 h prior to ovulation, the fertilization rates are significantly reduced when the insemination to ovulation interval is longer than 24 h (SOEDE et al., 1995; NISSEN et al., 1997; ARDON et al., 2003). In the pig, the functional reservoir is established in the caudal isthmus of the oviduct. VIRING and EINARSSON (1980), after inseminating gilts and then examining their oviducts, found the highest quantity of sperm cells in the lower part of the isthmus. It has since been established that the sperm reservoir occupies about 2 cm of the UTJ and caudal part of the oviducts (HUNTER 1984; RODRIGUEZ-MARTINEZ et al., 2005).

2.2.1 Function

The sperm reservoir is believed to have different functional purposes. Binding to the reservoir preserves the fertilization capacity of sperm (SUAREZ, 2001). It has been found that, as long as the sperm cells are in the reservoir, the motility remains depressed and the acrosomal membrane does not show changes (HUNTER, 1990; MBURU et al., 1996). Sperm maintain a
low metabolic rate, so that their limited metabolic reserves do not become exhausted before fertilization (HUNTER, 1984). Sperm viability is prolonged in bound sperm (TÖPFER-PETERSEN et al., 2002). THIBAULT et al. (1975) observed that the bovine spermatozoa accumulated mostly in the longitudinal non-coiled folds of the utero-tubal junction. In these folds, no leukocytic invasion was observed, which might be an important factor in sperm cell survival.

The reservoir is also believed to regulate the physiological state of the sperm, specifically the processes of capacitation and motility hyperactivation (RODRIGUEZ-MARTINEZ et al., 2001; SUAREZ, 2002). Capacitation is initiated by the uptake of Ca$^{2+}$ by the sperm cell; this uptake is depressed in oviduct bound sperm (TÖPFER-PETERSEN et al., 2002). Another capacitation-related event, the tyrosine phosphorylation, is also suppressed while sperm are bound to the oviduct (TÖPFER-PETERSEN et al., 2002). Motility hyperactivation, a process independent of the acrosome reaction, is a change in sperm flagellar movement (observed in vitro) that involves a significant increase in flagellar bend amplitude and, normally, in beat asymmetry, as well (SUAREZ, 2001). The regulation of capacitation and motility hyperactivation would ensure that the sperm are in the proper physiological state when ovulation occurs (SUAREZ, 2002). Thus, the two gametes would be able to meet at the site of fertilization at the right time (WAGNER et al., 2002).

It is also believed that the reservoir plays an important role in the prevention of polyspermy, by allowing only a few sperm to reach the fertilization site at a time (SUAREZ, 1998). When the reservoir is bypassed, there is increased incidence of polyspermy in the pig (HUNTER and NICHOL, 1988). Based on in vivo studies of distribution of sperm in the ovaries that show a gradient in the number of sperm reaching the site of ovulation, MBURU et al. (1996, 1997) have proposed that the sperm reservoir continuously releases small groups of sperm instead of triggering a massive release in the peri-ovulatory period. Because the life span of capacitated spermatozoa is short, this process would allow continuous presence of capacitated spermatozoa in the upper oviduct, ready to fertilize the oocytes once ovulation occurs (RODRIGUEZ-MARTINEZ et al., 2005).
2.2.2 Establishment and maintenance

The first sperm arrive at the functional reservoir as early as 15-30 min after AI and a population of sperm adequate for fertilization is found there within 1-2 h (HUNTER, 1981; 1990). The number of sperm cells in the reservoir remains relatively stable until roughly 24 h after insemination (FIRST et al., 1968). During the next 24 h, the reservoir’s population declines to the point that, by the time the embryos pass into the uterus, as early as 48 h after ovulation, there are no longer any spermatozoa at this site (RIGBY, 1966; HUNTER, 1988; ROZEBOOM et al., 2000).

Binding of the sperm to the oviductal epithelia is believed to be carbohydrate-mediated (TÖPFER-PETERSEN, 1999; SUAREZ, 2001). Both sperm and oviductal epithelia have ligands (oligosaccharides) and complementary receptors (lectins) that are involved in the sperm-oviduct binding in vitro (TÖPFER-PETERSEN et al., 2002). The spermadhesins of the sperm surface are believed to be the molecules that bind to the receptors of the epithelial carbohydrates (TÖPFER-PETERSEN et al., 2002). The carbohydrates involved in the binding of sperm to the oviduct are species specific: fetuin and sialic acid in the hamster (DEMOTT et al., 1995), galactose in the horse (LEFEBVRE et al., 1995), fucose in cattle (LEFEBVRE et al., 1997), and both ovalbumin and mannopentaose in the pig (TÖPFER-PETERSEN et al., 2002).

The binding of spermatozoa to the epithelium might be aided by the physiological characteristics of the isthmus, including a narrow lumen, which is caused by edematization of the epithelium. SUAREZ et al. (1997) hypothesized that, in bovines, a viscous, sticky secretion of mucus in the lumen of the reservoir could hinder sperm progress in the tract. Both a narrow lumen and the stickiness of the mucus would force the sperm to swim slowly against the mucosal surface of the oviduct, thus increasing their contact with potential binding sites (SUAREZ, 2001). It should also be noted that ciliation is more dense during estrus (BUHI et al., 1997), which narrows the lumen further (SUAREZ, 2001). MBURU et al. (1997) found that the epithelium-bound spermatozoa, especially the head, maintained close contact with the microvilli and cilia. The authors concluded that, in order to survive inside the oviduct, sperm has to maintain a continuous the attachment to the epithelia.
2.2.3 Sperm release

Sperm must detach from the oviductal epithelia to be able to reach the fertilization site and interact with the oocyte. The specific mechanism of sperm release is not completely understood. It is believed, however, that the release occurs when sperm plasma membrane changes producing a loss of carbohydrate binding affinity to the oviductal epithelium. These changes are attributed to the initiation of capacitation (SUAREZ, 1998; GUALTIERI and TALEVI, 2000). This loss of affinity appears to be a sperm-related event, as epithelia do not lose their sperm-binding capacity upon sperm release in vitro (GUALTIERI and TALEVI, 2000). SUAREZ and HO (2003) have suggested that an independent phenomenon, hyperactivation, might provide increased pulling force to aid in this detachment.

Several researchers concur in their belief that sperm release is a peri-ovulatory event (HUNTER et al., 1972; MBURU et al., 1996, 1997; SUAREZ, 1998; GUALTIERI and TALEVI, 2000; TIENTHAI et al., 2004). It is believed that the release might be a result of pre-ovulatory secretion of progesterone by the Graafian follicles (HUNTER et al., 1972) or of the initiation of capacitation (SUAREZ, 1998; GUALTIERI and TALEVI, 2000). It is also believed that sperm are continuously released in small numbers (TIENTHAI et al., 2004; RODRIGUEZ-MARTINEZ et al., 2005). A continuous flow of small batches of sperm would ensure that a few capacitated sperm are constantly available in the upper oviduct, awaiting ovulation (RODRIGUEZ-MARTINEZ et al., 2005).

2.3 Sperm chromatin

Sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins (WARD and ZALENSKY, 1996). The sperm chromatin differs markedly from that found in somatic cell nuclei, as will be explained below.

2.3.1 Sperm chromatin structure

The chromatin of mature mammalian sperm is an extremely compact and stable structure: the chromatin in sperm is nearly 6 times as condensed as that in somatic cells (POGANY et al., 1981; SAKKAS et al., 1999). Such condensation makes the sperm DNA transcriptionally
inert, structurally compact and protected (ARAVINDAN et al., 1997). In somatic cells, the DNA is linked by histones and coiled first into nucleosomes and then into solenoids (Figure 3). This arrangement makes the DNA negatively supercoiled, meaning that the supercoils induced by the histones are in a direction that would unwind the double helix if the bases were separated (WARD and COFFEY, 1991). During spermiogenesis, the histones that link DNA in somatic cells are replaced by protamines. These protamines contain thiols (SH), which are oxidized to disulfides (SS) during sperm maturation in the epididymis (KOSOWER et al., 1992). The protamines are bound to the DNA in the minor groove of the DNA strand; this protamine-DNA complex fits into the major groove of a neighboring DNA strand in a way that allows the DNA strands to be packed side-by-side in a linear array and not supercoiled (WARD and COFFEY, 1991). Inter- and intramolecular covalent disulfide bonds between the protamines stabilize the chromatin (Figure 3). During the re-arrangement of chromatin, transient, endogenous DNA strand breaks (also called “nicks”) appear. The function of these strand breaks appears to be the elimination of DNA supercoiling. Those DNA strand breaks, or nicks, are repaired during sperm maturation. The mechanisms of formation and repair, however, are poorly understood (MARCON and BOISSONNEAULT, 2004). The strand breaks disappear once protamination is complete (WARD and COFFEY, 1991). The organization of DNA within the sperm head is necessary for proper DNA replication (WARD and COFFEY, 1991).

At fertilization, the oocyte must remodel the condensed sperm chromatin into its accessible, transcriptionally active form (MCLAY and CLARKE, 2003). This remodelling of the chromatin involves the replacement of protamines by histones (MCLAY and CLARKE, 2003). The chromatin will finish its decondensation process once the histones are coiled into nucleosomes (MCLAY and CLARKE, 2003). The decondensation gives the sperm head a “twisted” appearance (HUNTER and DZIUUK, 1968), making it swell slightly. The transfiguration of the sperm head from a slightly swollen stage to an early pronucleous with an intact membrane requires approximately 1.5-2 h (HUNTER, 1972).
Figure 3. DNA packaging structures in somatic vs. sperm nuclei. In the somatic nucleus, the DNA is linked by a nucleosome consisting of histone octamers; this induces a negative supercoiling of the DNA. In the sperm nucleus, on the other hand, the DNA is linked by protamines, which do not induce supercoiling. The sperm DNA is stacked and requires less volume than the DNA in somatic cells. (From WARD and COFFEY, 1991).

2.3.2 Origin of chromatin damage

Defective spermatogenesis. Spermatogenesis might be disturbed by physiological and environmental stress as well as by gene mutations and chromosomal abnormalities (EVENSON et al., 2002). In humans, it has been observed that AIDS, in its early stages, has disturbed spermatogenesis and caused DNA damage in the ejaculate (AHMADI and NG,
Varicocele, chryptochidia and orchitis, all of which may disturb spermatogenesis, may also result in unstable sperm chromatin structure (FORESTA et al., 1989). Heat stress also affects spermatogenesis and causes an unstable chromatin structure. Several studies in mice report a reduction in the number and development of post-implantation embryos or in the development of embryos in vitro after induction of heat stress caused either by scrotal heating (JANNES et al., 1998; SETCHELL et al., 1998) or by ambient temperatures of 36 °C (ZHU et al., 2004). A similar effect was observed in men after periods of illness-induced fever (EVENSON et al., 2000). Thermal insult induced by scrotal insulation increased the percentage of chromatin instability in bulls (ACEVEDO, 2001).

When the chromatin packaging is disturbed because of a deficient replacement of histones by protamines (AUGER et al., 1990; MANICARDI et al., 1995), the resulting sperm could have increased DNA instability and sensitivity to denaturing stress (AHMADI and NG, 1999). Problems in the nuclear remodelling process in the later stages of spermatogenesis could also produce DNA damage (SAKKAS et al., 2002). A defective spermatogenesis could also account for the release of immature sperm (FISCHER et al., 2003). The presence of endogenous DNA strand breaks in ejaculated sperm is a sign of incomplete maturation during spermiogenesis and shows underprotamination and decondensation of the chromatin structure (MANICARDI et al., 1995; SAKKAS et al., 1999).

**Apoptosis.** It is believed that testicular germ cell apoptosis (i.e. programmed cell death) occurs physiologically and continuously during spermiogenesis (SAKKAS et al., 1999). FISCHER et al. (2003) stated that when the apoptosis process is defective, the sperm cells where an incomplete apoptosis took place might appear in the ejaculate. These sperm cells have damaged DNA (FISCHER et al., 2003) and will contribute to poor sperm quality (MOUSTAFA et al., 2004). MOUSTAFA et al. (2004) found a correlation between the presence of cells expressing apoptotic markers and chromatin instability. The authors proposed that DNA damage arose to a certain extent because of a defective apoptosis during spermiogenesis, but mainly from reactive oxygen species (ROS) production. SAKKAS et al. (2002), who also found a relationship between these kinds of defects in ejaculates, suggested that the presence in the ejaculate of sperm with apoptotic markers could be due to a defective remodeling of the cytoplasm during spermatogenesis, while the presence of DNA damage could arise from defective protamination during spermiogenesis.
Reactive oxygen species (ROS). ROS attack the double bonds associated with unsaturated fatty acids, initiating a lipid peroxidation chain reaction that will lead to a loss of membrane fluidity and a subsequent loss of sperm function (AITKEN and KRAUSZ, 2001). ROS have been shown to impair DNA integrity and fertilizing capacity (FISCHER et al., 2003; MOUSTAFA et al., 2004). Both mammalian sperm (AITKEN et al, 1997; ECROYD et al., 2003; MOUSTAFA et al., 2004) and the leukocytes present in the ejaculate (AITKEN et al., 1992) produce ROS. The biochemical mechanisms involved in the production of ROS are poorly understood, but ROS production is believed to be a byproduct of metabolic processes, seems to be induced by exogenous NADPH oxidase and might be related to capacitation (AITKEN et al., 1997; ECROYD et al., 2003). ROS generation is high in ejaculates with high percentages of cytoplasmic droplets, which could explain the correlation between cytoplasmic droplets and DNA denaturation in sperm (FISCHER et al., 2003).

Environmental toxins and radiation. The effect of environmental toxins has been studied extensively in humans. Cigarette smoke, for example, increases oxidative DNA damage in human sperm cells (SPANO et al., 1998; AHMADI and NG, 1999). Other toxins studied include those originating from pollution or industrial chemicals (AHMADI and NG, 1999). Air pollution has proven to have a detrimental effect on chromatin stability (SELEVAN et al., 2000). Some chemicals and medications, such as the chemotherapeutic drug cyclophosphamide (CODDINGTON et al., 2004), and organophosphorous pesticides (CONTRERAS et al., 1999; SANCHEZ-PENA et al., 2004) disrupt some step of the chromatin condensation, thus affecting chromatin structure. Hydroxyurea was found to disrupt DNA synthesis, thus altering sperm chromatin structure (EVENSON and JOST, 1993). Gamma radiation damaged the DNA and, although affected sperm could fertilize, the embryo development was hindered (AHMADI and NG, 1999). X-rays affected the sperm chromatin structure in mice (SAILER et al., 1995).

Age of donor and abstinence period. Chromatin structure stability improved with age in bulls (KARABINUS et al., 1990) but decreased in humans, rabbits and mice (SPANO et al., 1998; GOGOL et al., 2002; SINGH et al., 2003, respectively). SPANO et al. (1998) observed that chromatin instability increased with longer periods of abstinence in men, probably because of the over maturation of sperm in the epididymis; other studies have not found such a relation (EVENSON et al., 1991).
Storage length and temperature. LO et al. (2002) found that the temperature of semen storage in vitro, more than the storage length, affected the chromatin stability of stallion sperm. When sperm was stored at 5 °C, the chromatin quality remained unchanged for up to 46 h, but with storage temperatures of 37 °C the percentage of sperm with unstable chromatin increased significantly. ESTOP et al. (1993) found a similar decline in the chromatin stability in the sperm of mice incubated for up to 48 h.

2.3.3 Sperm chromatin effect on fertility and embryo development

The effect of sperm chromatin on fertility increases with the use of advanced assisted reproductive techniques, such as IVF or intracytoplasmic sperm injection (ICSI), because in this case sperm selection is largely a random process that may result in the inadvertent use of DNA-damaged sperm (FISCHER et al., 2003). LIU and BAKER (1992) found that human semen without severe defects in sperm morphology and with normal sperm-zona pellucida binding may fail to fertilize because of abnormal nuclear chromatin. HOSHI et al. (1996) observed that human semen samples that poorly fertilized oocytes in IVF systems had low chromatin stability. These sperm fertilized successfully only when used in ICSI. This result concurs with the conclusions of BUNGUM et al. (2004). LOPES et al. (1998), however, determined that the percentage of sperm with DNA fragmentation was negatively correlated with the fertilization rates obtained after ICSI. Chromatin abnormalities were also suggested as a cause of failure in the formation of the male pronucleus after ICSI (LEE et al., 2003). DNA-damaged sperm used for ICSI may impede the initiation or completion of decondensation, leading to a failure of fertilization and the finding of condensed sperm in unfertilized oocytes (SAKKAS et al., 1999).

Certain sperm defects might signal incompetence not only in the defective sperm but also in the apparently normal sperm of the ejaculate; this incompetence will impair embryonic development. Such an effect has been observed in ejaculates containing sperm with abnormal chromosomes (THUNDATHIL et al., 2000). EVENSON et al. (1999) referred to the sperm showing a certain defect as the “tip of an iceberg”: although only a few sperm show the defect, the rest of the ejaculate could have the same defect but to a lesser, non-detectable degree. This means that apparently normal sperm in abnormal ejaculates are most likely the source of male-related early embryonic death (SAACKE, 2004).
2.3.4 Evaluation of chromatin structure instability

Assays used for the evaluation of sperm chromatin are varied and include the single cell gel electrophoresis assay (COMET), the terminal desoxynucleotidyl transferase-mediated nick end labeling (TUNEL), the \textit{in situ} nick translation test (NT), the sperm chromatin structure assay (SCSA) and the acridine orange test (AOT) (reviewed by EVENSON et al., 2002). The stains used include aniline blue (AB), which signals persistency of histamines (FORESTA et al., 1992), Chromomycin A3 (CMA 3), which shows protamine deficiency (BIANCHI et al., 1993), and acridine orange (AO), which helps to distinguish single and double stranded nucleic acids (TEJADA et al., 1984).

In 1980, EVENSON et al. developed the Sperm Chromatin Structure Assay (SCSA). This test evaluates the resistance of sperm chromatin to thermal- or acid-induced denaturation \textit{in situ} (EVENSON et al., 1980; BALLACHEY et al., 1988). Under the SCSA conditions, abnormal chromatin structure is defined as the increased susceptibility of sperm DNA to acid-induced denaturation \textit{in situ} (EVENSON et al., 2002). This assay uses acridine orange (C$_{17}$H$_{20}$N$_{3}$Cl) to differentiate sperm with stable and unstable chromatin structure. Acridine orange (AO) is a metachromatic stain that can detect single and double stranded nucleic acids. It interacts differently with polynucleotides, depending on how it attaches to them. When the DNA or RNA is double stranded, AO intercalates between their stacked bases and the stain will fluoresce green at 530 nm. When the nucleotide is predominantly single-stranded, AO binds to the phosphate backbone to produce a stacked array and the stain will fluoresce red at 640 nm. (MACINNES and URETZ, 1966; KOSOWER et al., 1992; SHELTON SCIENTIFIC, 2004). The SCSA consists of subjecting an aliquot of sperm to acid-induced denaturation; then, the sperm cells are stained with AO. The samples are then submitted to flow cytometry, where 5000 sperm per sample are analyzed and the results are represented in a histogram. (EVENSON et al., 1994). The extent of DNA denaturation is quantified by the ratio of red to total (green + red) fluorescence, originally called alpha t ($\alpha_t$) (DARZYNKIEWICZ et al., 1975). Of these, the most important variables of $\alpha_t$ measured are COMP $\alpha_t$ (percentage of cells outside the main population of $\alpha_t$), which measures the cells with abnormal chromatin structure, and the standard deviation of $\alpha_t$ (SD $\alpha_t$), which measures the extent of the abnormality (EVENSON et al., 1994). Recently, the term $\alpha_t$ was replaced by DNA Fragmentation Index (DFI); the variables obtained include the percentage of high DNA
stainability (HDS), as well as both the standard deviation and the mean of the DFI (EVENSON et al., 2002)

In 1984, TEJADA et al. developed the first modification of SCSA for conventional fluorescent microscopy. Their motivation was that the SCSA, a flow-cytometric analysis, requires specialized equipment, which was very expensive at the time (TEJADA et al., 1984). The authors called this new test the acridine orange test (AOT). In this test, instead of examining the semen as a liquid, sperm is smeared on slides, where it is subjected to acid-induced denaturation and subsequent staining with AO. Another difference to the flow cytometric method is that it is based on human visual interpretation of the fluorescence. The slides are observed and evaluated under a fluorescent microscope using a 490-nm excitation filter and a 530-nm barrier filter (TEJADA et al., 1984). Using this method, TEJADA et al. (1984) found that the color of the “red” cells ranges from yellow to red, while the green was quite discernible from the other colors. The authors determined that 300 cells were necessary to obtain reproducible evaluations. This method has been criticized because of the range of colors found, the rapidly fading fluorescence and the heterogeneous slide staining (DURAN et al., 1998; EVENSON et al., 1999). The correlation between the results obtained through the AOT and the SCSA methods is low; for this reason, EVENSON et al. (2002) regarded the AOT as imprecise.

Using a modified fluorescent microscope AOT, KOSOWER et al. (1992) found that the protamines of normal, mature sperm cannot be sufficiently dissociated from DNA without prior reduction of the disulfide bonds (SS) to thiols (SH). Their results indicate that, when the nuclear protamines of the sperm are rich in disulfide bonds, the sperm fluoresce green after acid treatment. Conversely, when the protamines are poor in disulfide bonds, the sperm will fluoresce red. The reduction of the disulfide bonds, and the protection of thiols, can be achieved using 1,4-dithiothreitol (DTT) (KOSOWER et al., 1992).

ACEVEDO (2001) suggested that the use of thiol-protectant chemicals, like DTT or 2-Mercaptoethanol (2-ME), could help stabilize the chromatin after acid treatment, thus minimizing the problems related to the AOT technique, i.e. rapid fading of the fluorescence and/or shifts in fluorescence color over time. ACEVEDO (2001) combined the methods of EVENSON’s group (BALLACHEY et al., 1988), TEJADA et al. (1984) and KOSOWER et al. (1992) and developed a method called Acridine Orange Acevedo Chromatin Structure
Assay (AOA). ACEVEDO (2001) used a lower DTT concentration than that used by
KOSOWER et al. (1992) (1 vs. 5 mMol/l). This lower concentration still facilitates the entry
of AO to the nucleus (LÖHMER, 2003). A problem with the AOA is that the stain still fades
within a short period of time, leading to inconsistent staining and difficult evaluation of the
sperm cells (ACEVEDO, 2001; LÖHMER, 2003). ACEVEDO (2001) further modified this
technique by using 2-ME instead of DTT; 2-ME is included in most of the steps of the assay.
Even with this modification, ACEVEDO (2001) found that the slides should be evaluated
within 10 minutes of the AO staining. WABERSKI et al. (2002) modified the DTT-AOA
method to improve the DNA staining so that the stain would remain visible and clear longer.
Dimethylsulfoxide anhydrous (DMSO) was added to the DTT solution; DMSO increases the
permeability of the cell membrane, thus facilitating the entrance of DTT into the sperm
nucleus. The times used for the acid treatment and for the AO staining were also slightly
modified. The modifications were found to improve the evaluation of sperm cells, both in the
consistency within and between slides and in the length of time the stain remained fast. The
evaluation of 500 cells was found to yield reproducible results. The method developed by
WABERSKI et al. (2002) was called the modified fluorescent microscopic Sperm Chromatin
Structure Assay (mfSCSA). It was further refined by LÖHMER (2003) and by VOLKER
(2004) for its use in bovine and porcine sperm, respectively. WABERSKI et al. (2002)
compared this fluorescent microscopic method to the flow cytometric SCSA described by
EVENSON et al. (1980) and found that they were strongly correlated (r = 0.95, P < 0.01).
3 Materials and Methods

3.1 Equipment, chemicals and solutions

All equipment, chemicals and solutions are listed in the appendix.

3.2 Prevalence and persistency of chromatin instability in boar semen

3.2.1 Semen

Semen from 173 healthy, fertile boars from a commercial artificial insemination station was used. The semen was delivered overnight using an express delivery service. The semen doses were diluted in Beltsville Thawing Solution (BTS) (PURSEL and JOHNSON, 1975) and contained a total of $2 \times 10^9$ sperm cells.

3.2.2 Modified fluorescent microscopic Sperm Chromatin Structure Assay (mfSCSA)

3.2.2.1 Washing of samples and preparation of slides

First, four ml of each semen sample were placed in centrifuge tubes. Then, 2 ml of buffer solution were added and the samples were centrifuged at 2100 g for 10 minutes. The supernatant was removed and 2 ml of buffer solution were added. The samples were mixed thoroughly and centrifuged once again at 2100 g for 10 minutes. The supernatant was removed, but a small amount of the liquid was left for resuspension of the sperm pallet. Next, a small droplet (ca. 10 µl) was placed on a Superfrost® Plus slide and smeared using another slide. The slide was air-dried for at least 20 minutes and kept in refrigeration until its further processing (normally the same day; in rare cases, the following day).

3.2.2.2 Disulfide reduction (chromatin decondensation)

This step took place under an extractor at room temperature. The slides were first placed horizontally on test tubes racks, taking care that they were as level as possible. Then, each slide was completely covered with 2 ml of DTT/DMSO solution. They were left to react for 30 minutes. Then, each slide was washed with buffer solution using a wash bottle and placed
in a Hellendahl vertical glass stain jar previously filled with 30 ml of buffer solution. The slides were left in the jar for 10 minutes, then taken out, wiped once on the sides and back with absorbent paper and placed vertically against the test tube racks to air-dry ca. 20 minutes.

3.2.2.3 Chromatin denaturation

This step took place at room temperature under an extractor, while preventing direct contact of the samples with light. Carnoy’s solution was prepared in a vertical Hellendahl glass stain jar while the slides were being air-dried. The jar was covered with aluminum foil. The air-dried slides were placed into the jar and left to react for 100 minutes. Then they were taken out, wiped once on the sides and back with absorbent paper and placed vertically against the test tube racks to air-dry for at least 10 minutes.

3.2.2.4 Chromatin staining

During this step, direct contact of the samples with light was avoided. A pre-cooled, vertical Hellendahl glass stain jar was placed into a water bath, also pre-cooled. The acridine orange staining solution was prepared in the stain jar. The slides were placed into the jar and left for 20 minutes at 4 °C. Then, the slides were taken out, wiped once on the sides and back with absorbent paper and placed in a pre-cooled jar filled with 30 ml of buffer solution. They were left in the buffer solution for 10 min at 4 °C. Then, they were taken out, wiped again, and placed vertically against the test tube racks to air-dry for at least 20 minutes. Once the slides were dry, they were placed in a slide storage box at 4 °C until evaluation. Evaluation normally took place the same day, or, rarely, the following day.

A control sample of sperm, which had been frozen, was processed with each staining batch to assure that the factors related to each work day, such as room temperature and humidity, would not influence the results. The room temperature and humidity prevalent each working day, as well as any abnormal circumstances, were noted.
3.2.2.5 Setup of the fluorescent microscope and AnalySIS software

The fluorescent microscope was set to x 200, phase 2; 450-490 nm filter, FT 510, LP 520. The analySIS computer software was used for the evaluation.

The program was set to recognize as sperm only particles bigger than 700 pixels. This setting prevents the detection of small dust particles as sperm.

The evaluation recognizes two possible classes of DNA: single-stranded and double-stranded. The classification is based on the fluorescence acquired by the sperm cell after acridine orange staining. Acridine orange intercalates between the stacked bases of double stranded DNA and fluoresces green at 530 nm. In the case of single-stranded DNA, acridine orange binds to the phosphate backbone of the nucleotide to produce a stacked array and fluoresces red at 640 nm. Class one were single-stranded DNA (ssDNA), which show red fluorescence, and class two were double-stranded DNA (dsDNA), with green fluorescence. The sperm were divided into the two classes according to the quantity of single- or double-stranded DNA (ssDNA and dsDNA) found in each of them.

Sperm cells are placed under the microscope and seen through a “live-window” in a computer monitor. In this live window, the exposure time for the digital camera was set at ca. 40 ms, the light sensitivity was set to ISO 200 and the correction of the color scale was set to 0.9, 0.9 and 1.57 for red, green and blue, respectively. This correction is needed because some cameras alter the real colors of the photographed subject. Once a field was selected for evaluation, it was photographed using the “Super High Quality” (2776 x 2074 pixel) feature. After a picture was taken, it appeared in a second computer monitor, enlarged by 56%.

Next, the amount of ssDNA and dsDNA per sperm cell was determined. At least one red and one green sperm cell in a picture were selected; then, their intensity profile was evaluated. This information was used to choose the threshold values for each fluorescence channel needed for the classification of the sperm (blue, red and green). These threshold values referred to the quantity of blue, red and green color found in both “green” and “red” sperm. After the threshold values were chosen, the sperm was marked as either green or red. An optimal set of threshold values would be one where all sperm were satisfactorily covered. The normal amount of green and red in a given sperm is 2/3 red and 1/3 green in the case of “red”
sperm and 1/2 green and 1/2 red in “green” sperm. It was seen, however, that in certain slides the population of sperm tended to be either reddish or greenish. In these, the threshold values needed to be modified accordingly. Because of this, the selection of the threshold values was a crucial part of the sperm evaluation.

Once the threshold values for each fluorescence channel were selected, they were used for all pictures taken from a given slide. During the evaluation, sperm were categorized as class one (ssDNA) or two (dsDNA). Then, the results were summarized per class. The results obtained were: the number and percentage of “red” and “green” sperm, the mean wavelengths of the green and red portions found in each class, and the intensity and saturation of both green and red colorings per class. From this data, the mean red coloring found in “green” sperm was calculated by multiplying the mean red value by the mean saturation of color and dividing the result by the mean color intensity. This information served as a quality control parameter, as it would indicate how “greenish” or “reddish” a particular slide was.

### 3.2.2.6 Evaluation of semen samples

At least 4 fields were photographed per slide. The number of photographed fields varied depending on the number of sperm cells in each picture, because a minimum of 500 sperm cells per slide were to be evaluated. The same set of threshold values for each fluorescence channel was used to evaluate all photographed fields on a slide.

### 3.2.3 Statistical analysis

The first semen sample taken from the boars was used to evaluate the prevalence of high percentages of chromatin instability in semen samples. The boars were divided into two groups (group 1 = “low” (<5%) chromatin instability, group 2 = “high” (≥5%) chromatin instability). The constancy of chromatin instability was evaluated by studying those boars whose semen showed “high” percentages of sperm with unstable chromatin in at least one of three consecutive semen samples. The samples with more than 5% chromatin instability will be named “positive samples” in this study. The arithmetic means and standard deviation of sperm with unstable chromatin were determined using the Excel software. The statistical analyses were done using the Wilcoxon Test (NPar1Way procedure). The correlation between the percentage of sperm with unstable chromatin and both sperm motility and morphology.
was done using the Spearman Correlation test (Correl Procedure). All statistical analyses were done using the SAS software and the criterion for significance was $P < 0.05$.

3.3 Establishment of an assay for the study of chromatin instability in sperm bound to the oviductal epithelia in vitro

3.3.1 Dynamics of oviductal explant – sperm binding

3.3.1.1 Boars

Four healthy, fertile boars from the group of boars kept at the institute’s facilities were used.

3.3.1.2 Oviducts

Oviducts were recovered from multiparous sows ($n = 14$) after slaughter at the city slaughterhouse.

3.3.1.3 Preparation of oviductal explants

The oviductal explants were prepared based on the oviduct explant assay, as described by PETRUNKINA et al. (2001). Briefly, oviducts were collected and placed in 4 °C PBS at the city slaughterhouse and transported to the institute’s laboratory for immediate processing. Once in the laboratory, all tissues surrounding the oviducts were carefully removed and the oviduct was cut longitudinally using medium dissecting forceps and iris scissors. After straightening the oviduct as much as possible, it was placed on a paraffin wax-filled glass petri dish and fixated with needles. Then, $0.5 – 1 \text{ mm}^2$ pieces of the oviductal longitudinal folds were cut using micro-surgery forceps and the extra delicate mini-vannas iris spring scissors and placed in small petri dishes filled with TALP medium at 38 °C. Thereafter, these pieces were called explants (Figure 4). Separately, several slides were prepared by drawing 3 frames with silicon grease and then placing a 20-µl droplet of TALP medium into each frame. The explants were then placed, one per frame, on the slides and observed on a monitor attached to an inverse microscope. The explants were observed at a magnification of x 63 and
x 320. During this time, the slides were kept on a warm plate to avoid cooling of the explants. Only explants where strong ciliary movement was observed were used for the experiment. These explants were placed in small petri dishes (one per sow) and refrigerated until use. A total of 24 explants were needed per work day (12 per boar).

Figure 4. Preparation of oviductal explants.

3.3.1.4 Sperm preparation

Sperm from two boars were used 24 and 72 h after dilution in Androhep. Motile sperm were selected by Percoll gradient density centrifugation. First, one centrifuge tube per sample was placed at 45°. Then, 2 ml of 70% Percoll solution were placed in each tube and 4 ml of 35% Percoll solution were added carefully, to avoid mixing the two solutions. Next, 4 ml of Androhep-diluted semen were added. The sperm were centrifuged for 10 min at 170 g followed by 20 min at 600 g. After centrifugation, the supernatant, which contained the immobile sperm, was removed. The pellet was re-suspended in 500 µl of 38 °C Androhep without EDTA, because EDTA blocks the intake of calcium, thus acting as a membrane stabilizer (LEVIS, 2000) and preventing capacitation. The concentration of sperm was determined using a Thoma Neu counting chamber and the sperm was diluted to obtain a final concentration of $5 \times 10^3$ sperm per µl. The sperm suspension was placed in a 1.5-ml Eppendorf vial and kept warm.
3.3.1.5 Co-incubation of sperm and explants

First, the explants were taken out of refrigeration and distributed in six small petri dishes (two per petri dish, each from a different sow), each containing a 60 µl droplet of TALP medium at 38 °C. The explants and the sperm suspensions were placed separately in an incubator with 5% CO₂ at 38 °C. After an equilibration time of 5 minutes, 30 µl of the sperm suspension (15 x 10⁴ sperm) were added to each of the TALP media containing the explants. The first group of two explants and sperm was co-incubated for 5 minutes, the second for 15, the third for 30, the fourth for 60, the fifth for 90 and the sixth for 120 minutes.

3.3.1.6 Determination of Binding Index

One slide per incubation time was prepared by drawing two squares with silicon grease and placing 60 µl of TALP into each square. After each incubation interval, the explants were taken out of the incubator and washed by placing them in a 60 µl TALP medium droplet that had been placed in a small petri dish, and then shaking the petri dish side to side vigorously. The explants were then placed in a new TALP-medium droplet and shaken again. Next, each explant was placed inside one of the squares previously drawn on the slides and observed through a monitor attached to an inverse microscope. Then, the explants were placed under an inverted microscope and filmed. The explants were first filmed at a magnification of x 63, which allowed for each of the two halves of the explant to be filmed. Then, three areas showing epithelia were filmed at a magnification of x 256. These areas are hereafter called fragments. During the filming, the image was refocused continuously so that all sperm cells could be seen clearly and counted. For the sperm count, a transparent folio was placed on top of the monitor; then, a line was drawn on top of each sperm with a marker; finally, all lines were counted. The recorded images were then used for estimation of the size of each fragment. The fragment sizes were measured using the software program AIDA. In the AIDA program, one image per area was photographed; this image should show a clear contour. The contour was drawn using the mouse. After the scale was typed in, the program estimated the size automatically. Once both the size of the fragments and the number of bound sperm found in each fragment were known, the BI (defined as the mean number of bound sperm per 0.01 mm² of explant) was determined as follows (PETRUNKINA et al., 2001):
BI per explant = \( \frac{N_1 + N_2 + N_3}{S_1 + S_2 + S_3} \)
BI per incubation time = \( \frac{\text{BI explant 1} + \text{BI explant 2}}{2} \)

Where \( N \) = number of sperm found in each fragment
And \( S \) = surface area (in \( \text{mm}^2 \)) of each fragment.

**Figure 5. Explant for BI determination.** Drawing of an explant and the three explant fragments where sperm were counted; these areas were filmed and their surfaces measured.

### 3.3.1.7 Statistical analysis

The effect of the co-incubation time and that of the storage time (i.e, semen age) on the binding capacity of sperm was evaluated using the Wilcoxon test (Npar1Way procedure) of the SAS software. The criterion for significance was \( P < 0.05 \).
Figure 6. Flow Chart of the experiment to determine the dynamics of sperm-oviduct binding.

Collection of oviducts of two multiparous sows at the city Slaughterhouse

Transportation to the Lab at 4° in PBS

Semen collection, evaluation and dilution (2 boars)

Semen conservation @ 16° for 24 and 72 h

Percoll gradient centrifugation of semen; determination of sperm concentration

Preparation of six 1-ml vials with 5x10^6 sperm/ml per boar

Equilibration in CO₂ incubator for 5 minutes (simultaneous to explant equilibration)

Addition of 20 µl of sperm to each petri dish containing 2 explants

Equilibration of 2 explants, each from a different sow, in 60 µl TALP, in each of 6 petri dishes, in the CO₂ incubator for 5 minutes

Co-incubation of 2 explants (one per sow) with sperm (n = 100,000) for different time intervals:
1st petri dish: 5 minutes
2nd petri dish: 15 minutes
3rd petri dish: 30 minutes
4th petri dish: 60 minutes
5th petri dish: 90 minutes
6th petri dish: 120 minutes

Filming of explants and counting of sperm

Refrigeration until use (less than 2 h)

Equilibration in CO₂ incubator for 5 minutes (simultaneous to explant equilibration)

Addition of 20 µl of sperm to each petri dish containing 2 explants

Preparation of six 1-ml vials with 5x10^6 sperm/ml per boar

Equilibration in CO₂ incubator for 5 minutes (simultaneous to explant equilibration)

Addition of 20 µl of sperm to each petri dish containing 2 explants

Equilibration of 2 explants, each from a different sow, in 60 µl TALP, in each of 6 petri dishes, in the CO₂ incubator for 5 minutes

Co-incubation of 2 explants (one per sow) with sperm (n = 100,000) for different time intervals:
1st petri dish: 5 minutes
2nd petri dish: 15 minutes
3rd petri dish: 30 minutes
4th petri dish: 60 minutes
5th petri dish: 90 minutes
6th petri dish: 120 minutes

Filming of explants and counting of sperm

Determination of explant areas and binding index (BI) per co-incubation time.

Figure 6. Flow Chart of the experiment to determine the dynamics of sperm-oviduct binding.
3.3.2 Sperm release from the oviduct in vitro

3.3.2.1 Chemicals and solutions

Heparin, Tyrode medium, TALP, D(+)–Mannose, calcium ionophore A23187, Pronase and liquid nitrogen were tested. The complete description of the chemicals and solutions is found in the appendix.

3.3.2.2 Boars

Four healthy, fertile boars from the group of boars kept at the institute’s facilities were used.

3.3.2.3 Oviducts

Oviducts were recovered from multiparous sows after slaughter at the city slaughterhouse.

3.3.2.4 Oviductal Explant preparation

The oviducts were brought to the laboratory in 4°C PBS. Once in the laboratory, oviductal explants were prepared as described above (see 3.3.1.3).

3.3.2.5 Sperm preparation

Four ml of semen were subjected to Percoll gradient centrifugation as described above (see 3.3.1.4). Afterwards, the pellet formed was resuspended in 500 ml of Androhep without EDTA.

3.3.2.6 Co-incubation of sperm and oviductal explants

The sperm were co-incubated with the oviductal explants for 30 minutes, which was the co-incubation period where the highest BI was found in the previous experiment (dynamics of oviductal explant - sperm binding).
3.3.2.7 Incubation of oviduct-bound sperm in different solutions to induce sperm release

After the co-incubation of sperm and oviduct explants, the explants were washed twice, as described above (see 3.3.1.6), to eliminate all sperm loosely attached to the explant. This moment was set as time 0. The explants \((n = 4\) for the control and each solution per incubation time combination) were then incubated in the solutions. The incubation periods for each solution varied. The incubation time for D(+)−Mannose (0.05 and 0.1M) was 30 min. In TALP medium, the explants were incubated in a first trial for up to 120 min; in a second trial, for 4, 6 and 8 hours. In Tyrode medium the explants were incubated for 10, 15, 30, 60 and 120 minutes. In the heparin solution (10 µg/ml, 100 µg/ml and 500 µg/ml diluted in both TALP and Tyrode media), the explants were incubated for 10, 30, 60 and 120 minutes. Sperm were incubated in 5% Pronase solution for 10 minutes. The incubation time for calcium ionophore A23187 (1, 5 and 10 µM/10 ml TALP medium) was set at 15, 30 and 60 minutes.

The snap-freezing of explants was done by placing them, along with a small quantity of TALP medium, into Eppendorf vials and dropping them into liquid nitrogen. They were left there for 10 minutes and then thawed by placing them in 39 °C water.

3.3.2.8 Evaluation of the effectiveness of each solution or chemical’s sperm release

At least two control explants were filmed and their binding index was determined as described above (see 3.3.1.6). After the sperm incubation in the different solutions, at least two explants each per solution and incubation time were filmed and their binding index determined.

3.3.2.9 Effect of long-term incubation in TALP medium and of snap-freezing in liquid nitrogen on the chromatin structure stability of sperm

Sperm were subjected to the mfSCSA (see 3.2.2) after long-term incubation (4, 6 and 8 h) in TALP medium. The resulting percentage of chromatin instability was compared to that of sperm, from the same sample, that had been subjected only to Percoll gradient density centrifugation. This was done to assure that the long incubation period had not affected the chromatin status of the sperm. Similarly, sperm that was snap-frozen in liquid nitrogen was
compared to sperm, from the same sample, subjected only to Percoll gradient density centrifugation.

3.3.2.10 Statistical analysis

The BI of the control explants was compared to that of the experimental explants, per solution and incubation time group. The statistical analysis was done with the Wilcoxon test (Npar1Way procedure) in the SAS software.

3.4 Prevalence of chromatin instability in sperm bound to the oviductal epithelia in vitro

3.4.1 Boars

Four healthy, fertile boars from a commercial artificial insemination station were used. The semen was delivered overnight using an express delivery service. The sperm in the semen doses were diluted in BTS and contained a total of $2 \times 10^9$ sperm cells.

3.4.2 Oviducts

Oviducts were recovered from multiparous sows after slaughter at the city slaughterhouse.

3.4.3 Oviductal strip preparation

The oviducts were brought to the laboratory in 4°C PBS. Once in the laboratory, the oviducts were placed in a petri dish containing 4°C PBS. The oviducts were stripped of all remaining tissue around them and open longitudinally. Then, the oviducts were placed on a petri dish filled with paraffin wax and fixed with needles. To maximize the number of sperm bound and then released, the oviduct explant assay, described above (see 3.3.1.3 through 3.3.1.6), was modified. Instead of using oviductal explants, long pieces of the oviductal epithelia were cut using dissecting scissors and with the help of medium dissecting forceps. The pieces were cut carefully under a stereomicroscope in order to obtain as much of the oviduct epithelia as possible. Thereafter, these epithelia pieces were called oviductal strips (Figure 7). The oviductal strips (two, one each from a different sow) were then put in a small petri dish
containing 3.5 ml of TALP medium. The strips were refrigerated while the sperm were prepared.

3.4.4 Sperm preparation

To concentrate the sperm, 50 ml of the semen doses (containing $2 \times 10^9$ total sperm) were placed in 50-ml plastic centrifuge tubes and centrifuged at 170 g for 10 minutes. Four ml of the semen were left in the tube and resuspended. The semen was then subjected to Percoll gradient density centrifugation, as described above (see 3.3.1.4). The resulting sperm pellet was re-suspended in 500 µl Androhep without EDTA and placed in a 1.5-ml Eppendorf vial. All sperm cells obtained were co-incubated with the oviductal strips.

3.4.5 Co-incubation of sperm and oviductal strips

The Eppendorf vial containing the 500 µl of sperm, as well as the petri dish containing the oviductal strips in the TALP medium, were placed in a CO$_2$-chamber for 5 minutes for equilibration. Then, the 500 ml of sperm were added to the petri dish containing the oviductal strips. They were co-incubated for 30 minutes. After this time, the oviductal strips were taken out and washed. The wash consisted of fixing the strips with medium dissecting forceps, moving the strips vigorously around the petri dish, then placing them in another petri dish containing 4 ml of TALP and moving the strips around the petri dish again. This was done twice. Then, the oviductal strips, along with 0.5 to 1 ml of TALP medium, were placed in a 1.5-ml Eppendorf vial.
3.4.6 Sperm release

The oviductal strips were snap-frozen by dropping the Eppendorf vials into liquid nitrogen, where they remained for 10 minutes. Then, the vials were placed in water at 39 °C to thaw the oviductal strips. The oviductal strips were fixed with medium dissecting forceps to remove them from the Eppendorf vial. The remaining liquid was placed in a centrifuge tube.

3.4.7 Preparation, mfSCSA treatment and evaluation of sperm samples

The sperm samples consisted in 2 ml each of diluted semen and Percoll-treated sperm, as well as all of the sperm released from the oviduct. The samples were washed as detailed above (see 3.2.2.1). The slides were treated with the mfSCSA, as detailed above (see 3.2.2.2 to 3.2.2.6). 500 cells each of diluted semen and the Percoll-treated sperm were evaluated using the AnalySIS program. The oviduct-bound and released sperm were evaluated subjectively under a fluorescent microscope without the assistance of the AnalySIS program. The AnalySIS program was not used because of the low numbers of sperm (< 20 per field) and because of the presence of oviductal cells with unspecific fluorescence staining. All sperm cells found on a slide were counted using a manual cell counter.

3.4.8 Statistical analysis

The percentage of chromatin instability in diluted sperm was compared to that of the sperm treated with Percoll gradient density centrifugation and to that of the sperm bound to the oviductal epithelia and then released. In all cases, the analysis was done using the Wilcoxon Test (NPar1Way procedure) of the SAS software. The criterion for significance was $P < 0.05$. 
Figure 8. Flow chart for the determination of the prevalence of chromatin instability in sperm bound to the oviduct *in vitro*.
3.5 Prevalence of chromatin instability in accessory sperm

3.5.1 Animals

Three healthy, fertile boars from a commercial artificial insemination station were used. The semen was delivered overnight, using an express delivery service. The sperm in the semen doses were diluted in BTS and contained a total of $2 \times 10^9$ sperm cells.

A total of 14 gilts were bought from a local commercial farm and transported to the facilities of the Institute for Reproductive Medicine of the School of Veterinary Medicine Hannover when they were 6 to 8 months old and weighed at least 90 kilos. The gilts were housed in groups of two to three.

3.5.2 Semen evaluation

The semen was evaluated according to the standard guidelines followed by the andrology laboratory of the Institute for Reproductive Medicine. Upon receipt of semen, sperm motility and morphology were evaluated. Motility was assessed under a binocular phase-contrast microscope with heating stage (37 °C, magnification x 400). Morphological evaluation was done after fixation in formaldehyde citrate solution under a phase-contrast binocular microscope with mechanical stage (magnification x 1000). Two aliquots per semen dose were snap-frozen in liquid nitrogen for mfSCSA treatment; this treatment was done at the same time as that of the embryos sired with each boar. Additionally, the motility, morphology and chromatin instability of four samples each from Boar 6 and Boar 7 were evaluated at day 7.

3.5.3 Estrus detection

This experiment was done following the Hannover Gilt Model (HGM) guidelines (ARDON et al., 2003). Detecting for estrus—every 12±1 hours—was begun three days after the gilts entered the facility. In order to detect estrus, the gilts were brought from their pens to an area surrounded by 4 boars, where any redness and/or swelling of the vulva and whether the gilts showed any interest in the boars was noted. However, the gilts were only considered to be in estrus upon observation of the “standing reflex,” when the sow stands immobile, arches her
back and cocks her ears in response to feeling pressure on her back, thus allowing the mating and/or insemination to take place. Estrus beginning before the 15th day after transport was classified as post-stress estrus and none of the gilts was inseminated during such an estrus. Any gilt whose estrus began on the 15th day or thereafter was considered to be in an “eligible cycle,” during which she could be inseminated.

The onset of estrus was estimated to be the halfway point between the last time that the gilt did not show the standing reflex and the first time that she did. And, likewise, the end of estrus was considered to be the halfway point between the last time that the gilt accepted back pressure and the first time that she rejected it.

### 3.5.4 Insemination

The gilts were inseminated 12 h after first detection of eligible estrus and every 12 h thereafter, as long as the gilt showed the standing reflex. This was a modification from the HGM, where one-time insemination is used.

All gilts were brought to and kept in front of a boar for insemination, which was done using a spiral insemination catheter. This procedure lasted at least 5 minutes, during which time back pressure (generally accomplished by having a person mount the gilt) and clitoral stimulation were provided.

After insemination, the gilt was assigned a number, which was used to identify her during the evaluation of the embryos/ova recovered.

### 3.5.5 Detection of ovulation

Transabdominal ultrasound observation of the ovaries was performed as soon as the gilt’s estrus was observed and every 12±1 hours thereafter, until ovulation was detected. In order to perform the observations, the sows were brought to an examination crate, where they were fed during the procedure. A 5 mHz Sector Probe Ultrasound “Sonoline” was used to observe the ovaries; all observations were made by placing the ultrasound’s probe on the right side of the gilts and all findings were recorded (WEITZE et al., 1990). Ovulation was estimated to have
occurred halfway between when follicles were still found and when they were no longer visible.

3.5.6 Recovery and evaluation of oocytes and embryos

Three to five days after ovulation took place, the gilts were sent to the Hanover city slaughterhouse. There, their reproductive tracts were removed immediately after slaughter, put into Styrofoam boxes to keep the oviducts at 37 °C and brought back to the institute’s laboratory for immediate recovery and evaluation of embryos. The animals were slaughtered only Mondays, Wednesdays and Fridays, which are the slaughterhouse’s work days.

Once in the laboratory, the corpora lutea in each oviduct were counted. Then, the oviducts and uterine horns were flushed to recover the embryos and oocytes. The flushing was done as follows: 40 ml of sterile PBS warmed to 39 °C was injected through the ampullatory end of the oviduct using a knobbled needle and a 50-ml syringe. The oviduct was massaged and cut. The uterine horn was clamped shut both near the uterus body and a little below the utero-tubal junction. The uterus body was cut off. The entire uterine horn was then tilted back and forth 2-3 times so that the liquid would pick up the oocytes and embryos, thus facilitating the collection of both in siliconized beakers. The upper part of the uterine horn was then held above the beaker, allowing the liquid to fall gently onto the glass wall. The goal was to get most of the liquid out as quickly as possible while avoiding damaging the embryos or oocytes. The uterine horn was then gently manually massaged, so that the remaining liquid was collected. Next, approximately 8 ml of liquid was transferred into each of several new plastic petri dishes (at least 5 per side) that were also kept at 39°C and already marked with the sow’s number and the uterine horn side. After no more liquid could be poured out of the beaker, it was rinsed again with PBS and this liquid placed in another petri dish. Then, the petri dishes were left covered for a few minutes to allow for sedimentation of the embryos or oocytes.

After sedimentation, a stereomicroscope (30 to 60 x) was used to find the embryos and oocytes. Each petri dish was first examined for up to five minutes, in order to find as many embryos and oocytes as possible, while not lingering at any given dish. Using a micro-pipette, the embryos and oocytes were then transferred into a smaller petri dish (one per uterine horn) that contained enough PBS to cover its surface. After all petri dishes had gone through this first search, a second search for any remaining embryos or oocytes could then be conducted.
If the liquid poured into any one dish was too cloudy to allow searching, its contents could be divided into two dishes, and diluted with a small amount of PBS.

Again using the stereomicroscope, the morphology of each embryo or oocyte was evaluated. The data and pictures presented by HUNTER (1974, 1988) and by BRÜSSOW and KAUFFOLD (1989) were used to get standard measures of morphological characteristics.

The categories (based on WABERSKI et al., 1994) used for the classification were:

1. Very good/good: the embryo contains the expected blastomere numbers, with or without minor alterations in its morphology, such as a slightly uneven perivitelline space or slightly unevenly distributed or sized blastomeres.
2. Retarded/degenerated: the embryo is at least 24 hours behind in its expected development and/or has major alterations in its morphology, such as an uneven perivitelline space or unevenly distributed or sized blastomeres.
3. Oocyte: it is unfertilized and has no more than one nucleus. The accessory sperm cell count is generally 0, though 1 or 2 can be found.

Next, the embryos were placed on Superfrost® Plus Slides using a micropipette. The stability of the accessory sperm chromatin structure was evaluated using the mfSCSA. For this, the slides were air-dried and then refrigerated until treated (normally the same day; in exceptional cases, the following day). Up to 6 embryos of the same quality and from the same gilt were placed in the same slide.

3.5.7 Preparation, mfSCSA treatment and evaluation of sperm samples

Two ml of diluted semen were washed as detailed above (see 3.2.2.1). The sperm and the embryo slides were treated with the mfSCSA as detailed above (see 3.2.2.2 to 3.2.2.6). 500 sperm cells from the diluted sperm were evaluated using the AnalySIS program. The accessory sperm were evaluated subjectively under a fluorescent microscope without the assistance of the AnalySIS program. The AnalySIS program was not used because of low numbers of sperm (< 20 per field) and because of the presence of embryonic cells with unspecific fluorescence staining. All accessory sperm present on a slide were counted using a manual cell counter.
3.5.8 Statistical analysis

The percentage of chromatin instability in diluted sperm was compared to the accessory sperm per boar. The effect of the boars on fertilization rates and rates of normal embryos were evaluated. The effect of the embryo’s morphology on the percentage of unstable chromatin of the accessory sperm was analyzed as well. Additionally, the effect of storage on the sperm motility, morphology and chromatin stability was evaluated. All analyses were done using the Wilcoxon Test (NPar1Way procedure) of the SAS software. The criterion for significance was $P < 0.05$.

3.6 Accessory sperm: a bio-monitor of sperm fertilizing capacity in vivo

3.6.1 Animals

Three healthy, fertile boars from the group of boars kept at the Institute for Reproductive Medicine of the School of Veterinary Medicine Hannover were used. 94 gilts were available for this experiment. All gilts were bought from a local commercial farm and transported to the institute’s facilities when they were 6 to 8 months old and weighed at least 90 kilos. The gilts were housed in groups of two to four.

3.6.2 Semen collection

Each of the three boars was collected every three days, with a different boar being collected every day.

3.6.3 Semen evaluation

The semen was evaluated according to the standard guidelines followed by the andrology laboratory of the Institute for Reproductive Medicine. Sperm motility of raw semen was evaluated immediately after collection and in diluted semen at days 1, 3 and 5 under a binocular phase-contrast microscope with heating stage (37 °C, magnification x 400). Raw semen was assessed morphologically after fixation in formaldehyde citrate solution under a phase-contrast binocular microscope with mechanical stage (magnification x 1000); diluted semen was evaluated on the first and fifth day after dilution. For the sperm’s viability
evaluation, 500 µl of semen diluted in Tyrode’s salt solution (10 x 10⁶ sperm/ml) was incubated for 15 min with 0.27 µl SYBR 14 base solution and 2 µ PI base solution in a 36° dark chamber, then smeared onto a slide and air-dried in the chamber. Sperm were evaluated under a fluorescent, phase-contrast binocular microscope (Filter 490-520 nm, Phase 3, magnification x 1000). A total of 200 sperm cells were evaluated for the morphology and viability assessments. Semen was used for this trial only if it had less than 25% abnormal forms and more than 80% motile sperm.

3.6.4 Semen dilution and use

Semen was extended in BTS. 80 ml of diluted semen was placed into 100 ml semen bottles at a concentration of 8 x 10⁸ total sperm cells. The bottles were then stored at 17 °C. The semen doses were used up to 30 h after collection. Semen that was not used after 30 h was discarded.

3.6.5 Estrus and ovulation detection

Estrus and ovulation detection were done as described previously (see 5.2.2 and 3.5.4). In the present experiment, the Hannover Gilt Model (HGM) guidelines (ARDON et al., 2003) were followed.

3.6.6 Insemination

Artificial insemination was performed only during eligible estrus. The gilts were inseminated only once, between 12-18 hours after first detection of estrus. The insemination procedure was as described above (see 3.5.3).

3.6.7 Recovery and evaluation of oocytes and embryos

Three to five days after ovulation took place, the gilts were sent to the Hanover city slaughterhouse. The uteri were recovered after slaughter and brought to the laboratory. The embryo recovery and evaluation was done as described above (see 3.5.5).

After this evaluation, each embryo or oocyte was placed on a slide inside a circle that had been drawn with permanent marker and they were stained with dye (Hoechst 33342) at a ratio
of 1:1. A cover glass was then placed on top of each circle, with care being taken so that the structure would remain within the circle. Then the embryo or oocyte was evaluated under a fluorescent microscope (magnification x 200-400, G355 excitation filter, FT395 dichronic beam splitter and LP420 emission filter; WABERSKI et al., 1994), in order to count the accessory sperm cells and the blastomeres. Each embryo or oocyte was given an identification number tied to that of the gilt, so that all the data on embryo quality and accessory sperm cell count could be linked to her. The evaluation or classification of embryos was based on both the morphology of the embryo (see 3.5.5) and its developmental stage, as observed under the fluorescent microscope.

3.6.8 Statistical analysis

For this study, all embryos and oocytes found in a given sow were considered as a litter (HANCOCK, 1961). Such litters were cataloged into litter types depending on the kind of embryos or oocytes found in them. In this manner, litters containing only normal embryos were classified as type 1; litters with both normal and degenerated embryos, as type 2; those with both normal embryos and oocytes, as type 3; those with both kinds of embryos plus oocytes, as type 4; and those with oocytes only, as type 5. This allowed for the study of the relationship between accessory sperm and embryo quality, while considering the effect of different factors on a whole litter. Because the accessory sperm counts were skewed (WABERSKI et al., 1994; WEITZE et al., 1990), the embryos were divided into zona classes according to their accessory sperm count. In this fashion, zona class 1 comprised embryos with 0-1 accessory sperm; zona class 2, those with 2-10 accessory sperm; zona class 3, those with 11-29 accessory sperm; and zona class 4, those with 30 or more accessory sperm.

All data analyses were performed using the SAS software. Accessory sperm numbers and percentage of normal embryos were tested for normality within the categories of the variables to be analyzed for possible effects and found to be non-Gaussian and skewed. As normality was not achieved after log (x + 1) transformation, the analysis of the relationships of the boar, the litter type, and the embryo quality to the accessory sperm counts and those of the boar and the litter type to the percentage of normal embryos were done with the Wilcoxon test (NPar1Way procedure). The frequency of each zona class per boar, litter and embryo quality was analyzed with the Cochran-Armitage trend test (freq procedure). The criterion for significance was P < 0.05.
4 Results

4.1 Prevalence and persistency of chromatin instability in boar semen

4.1.1 Prevalence of sperm with unstable chromatin in semen samples from an AI boar population

The majority (85.5%) of the samples evaluated (n = 173, one per boar) showed less than 5% of sperm with unstable chromatin. The mean for all samples, including those with more than 5% of sperm with unstable chromatin, was 3.2% (Table 1).

<table>
<thead>
<tr>
<th>Chromatin instability group</th>
<th>Boars (n)</th>
<th>Boars (%)</th>
<th>Sperm with unstable chromatin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Low”</td>
<td>148</td>
<td>85.5</td>
<td>2.2±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>“High”</td>
<td>25</td>
<td>14.5</td>
<td>9.2±4.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>All boars</td>
<td>173</td>
<td>100.0</td>
<td>3.2±3.3</td>
</tr>
</tbody>
</table>

**Table 1. Sperm with unstable chromatin in boars used for artificial insemination.**<sup>1</sup> Boars were divided into those showing “low” (<5%) or “high” (≥5%) percentages of sperm with unstable chromatin. a,b: indicates significant differences (P < 0.05) within a column. Data shows means ± standard deviations.

4.1.2 Persistency of high percentages of sperm with unstable chromatin in semen samples from an AI boar population

Of 13 boars tested three consecutive times and whose semen samples had more than 5% of sperm with abnormal chromatin (i.e. positive samples) at least once, all samples of seven boars were positive. Two samples of two boars were positive and only one sample each from six boars was positive. The mean percentage of chromatin instability in positive samples in boars with three positive samples was significantly higher (P < 0.05) than in boars with either one or two positive samples (Table 2)
Table 2. Chromatin instability of sperm samples with high percentages (≥5%) of
unstable chromatin. Three consecutive semen doses per boar were tested. This analysis was
done using only boars whose semen had more than 5% of sperm with unstable chromatin at
least once. a,b: indicates significant differences within a column (P < 0.05). Data shows
means ± standard deviations. 1Positive samples: samples with more than 5% of sperm with
unstable chromatin.

4.1.3 Association between chromatin instability and sperm defects

The percentage of cytoplasmic droplets and the percentage of total abnormalities were
significantly higher (P < 0.01) in ejaculates with high (≥5%) percentages of sperm with
unstable chromatin than in those showing low (<5%) percentages (Table 3). The percentage
of sperm with unstable chromatin correlated significantly with the percentage of sperm with
cytoplasmic droplets (r = 0.44, P < 0.01; Figure 9) and with the percentage of total
abnormalities (r = 0.41, P < 0.01) (n = 128 ejaculates from 54 boars). No correlation was
found between the percentage of sperm with unstable chromatin and other standard sperm
parameters, i.e. motility and morphological abnormalities other than cytoplasmic droplets.
<table>
<thead>
<tr>
<th>Chromatin instability group</th>
<th>Low</th>
<th>High</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boars (n)</td>
<td>38</td>
<td>16</td>
<td>54</td>
</tr>
<tr>
<td>Ejaculates (n)</td>
<td>77</td>
<td>51</td>
<td>128</td>
</tr>
<tr>
<td>Sperm with unstable chromatin (%)</td>
<td>2.5±1.2\textsuperscript{a}</td>
<td>10.2±5.0\textsuperscript{b}</td>
<td>5.6±5.0</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>79.9</td>
<td>80.1</td>
<td>80.0</td>
</tr>
<tr>
<td>Total sperm with morphological abnormalities (%)</td>
<td>17.7±11.2\textsuperscript{a}</td>
<td>30.0±17.4\textsuperscript{b}</td>
<td>22.5±15.2</td>
</tr>
<tr>
<td>Morphological abnormalities, other than cytoplasmic droplets (%)</td>
<td>7.3±8.2</td>
<td>8.8±10.1</td>
<td>7.9±9.0</td>
</tr>
<tr>
<td>Cytoplasmic droplets (%)</td>
<td>12.3±10.4\textsuperscript{a}</td>
<td>25.3±18.3\textsuperscript{b}</td>
<td>17.5±15.4</td>
</tr>
</tbody>
</table>

Table 3. Motility and morphological abnormalities of sperm according to the chromatin instability. \textsuperscript{1}Ejaculates were divided into those showing low (<5%) or high (≥5%) percentages of sperm with unstable chromatin. \textsuperscript{a,b} indicate significant differences between the groups (P < 0.01).

![Figure 9. Correlation between percentages of sperm with unstable chromatin and of sperm with cytoplasmic droplets. (r = 0.44, P < 0.01). n = 128 ejaculates from 54 boars.](image-url)
Figure 10. Sperm after treatment with the mfSCSA. Green cells are sperm cells with double-stranded DNA; red cells are sperm cells with single-stranded DNA.

4.2 Establishment of an assay for the study of chromatin instability in sperm bound to the oviductal epithelia \textit{in vitro}

4.2.1 Dynamics of oviductal explant – sperm binding

The number of sperm bound to the oviduct (binding index; BI) varied with time, reaching a peak between 30 and 90 min for semen stored short-term (24 h) and at 30 min for semen stored long-term (72 h). The BI at 5, 15 and 90 min of incubation in TALP medium of semen stored for 24 h was significantly higher than that of semen stored for 72 h. The BI was also dependent on the boar and on the ejaculate, as can be seen in the standard deviation found in both storage periods (Figure 11).
Figure 11. Binding index (BI; means and standard deviations) of sperm bound to oviductal explants in TALP medium at different incubation lengths. BI: sperm bound per 0.01 mm². a,b,c: significant differences between incubation lengths (P < 0.05). n = 2 explants each for four boars per incubation time and semen storage period (24 and 72 h).

4.2.2 Sperm release from the oviduct in vitro

Snap-freezing of the oviductal explants in liquid nitrogen released all oviduct-bound sperm. Sperm subjected to Percoll gradient density centrifugation and sperm that had been snap-frozen in liquid nitrogen had similar percentages of sperm with unstable chromatin. With respect to the control (30 min co-incubation of sperm and oviductal epithelia), the mean binding index (BI) of sperm bound to the oviductal epithelia was significantly reduced (P < 0.05) after an additional 6 h of incubation in TALP medium. In turn, the BI was significantly lower (P < 0.05) after 8 h of incubation than after 6 h (BI = 6.0, 5.5 and 0.3 for incubations lasting 4, 6 and 8 h, respectively; BI of control = 16.5) (Figure 12). The percentage of chromatin instability of sperm incubated in TALP medium was 40% higher than that of sperm subjected to Percoll gradient density centrifugation.
Figure 12. Binding index (sperm number per 0.01 mm² of oviductal epithelia) found in sperm co-incubated with oviductal epithelia in TALP medium during different incubation lengths. Incubation of 0 h = control explants, where BI was measured after 30 min of co-incubation of sperm and oviductal epithelia. a,b,c: indicate significant differences among the incubation lengths (P < 0.05). (n = 4 explants per incubation length).

The other chemicals and solutions used (Heparin, Tyrode medium, D(+)-Mannose, Pronase and Calcium Ionophore A23187, Heparin plus Tyrode) reduced the BI neither consistently nor sufficiently in any of the incubation lengths or concentrations. (n = 4 explants per each combination of incubation length-concentration).

4.3 Prevalence of chromatin instability in sperm bound to the oviductal epithelia in vitro

4.3.1 Morphological evaluation of sperm used for the oviduct binding experiment

Boars 1 and 2 had similar percentages of cytoplasmic droplets and of total morphological abnormalities. In these two aspects, Boars 1 and 2 were significantly different (P < 0.05) from Boars 3 and 4, which were similar to each other. The percentage of sperm with unstable chromatin was different (P < 0.05) for all four boars; however, semen from Boars 1 and 2
showed less than 5% unstable chromatin and semen from Boars 3 and 4, more than 5%. The percentage of motile sperm and the percentage of morphological abnormalities, other than cytoplasmic droplets, were similar for all boars (Table 4).

<table>
<thead>
<tr>
<th></th>
<th>Boar 1</th>
<th>Boar 2</th>
<th>Boar 3</th>
<th>Boar 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculates (n)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sperm with unstable chromatin (%)</td>
<td>1.6±0.5a</td>
<td>2.4±0.6b</td>
<td>15.4±1.0c</td>
<td>8.4±2.4d</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>81.3±6.3a</td>
<td>81.3±2.5a</td>
<td>76.7±5.8a</td>
<td>81.7±2.9a</td>
</tr>
<tr>
<td>Total sperm with morphological abnormalities (%)</td>
<td>11.3±9.2a</td>
<td>8.0±3.9a</td>
<td>30.0±2.6b</td>
<td>32.7±4.6b</td>
</tr>
<tr>
<td>Morphological abnormalities, other than cytoplasmic droplets (%)</td>
<td>3.5±4.4a</td>
<td>2.8±2.2a</td>
<td>3.3±2.5a</td>
<td>1.0±1.7a</td>
</tr>
<tr>
<td>Cytoplasmic droplets (%)</td>
<td>7.8±5.0a</td>
<td>5.3±3.3a</td>
<td>26.7±1.5b</td>
<td>31.7±5.7b</td>
</tr>
</tbody>
</table>

Table 4. Motility, morphology and chromatin instability of sperm used for sperm binding experiment. Sperm was evaluated after 24 h of storage. a,b,c = indicate significant differences (P < 0.05).

Chromatin instability correlated with the percentage of cytoplasmic droplets (r = 0.59, P = 0.02) and of total abnormalities (r = 0.70, P < 0.01)

4.3.2 Chromatin instability in diluted, Percoll-treated and oviduct-bound sperm

Significantly lower (P < 0.05) percentages of chromatin instability were found in sperm released from the oviduct than in sperm either diluted in Androhep or subjected to Percoll gradient centrifugation. Percoll gradient centrifugation also significantly reduced (P < 0.05) the percentage of sperm with unstable chromatin in two boars (Figure 13).
Figure 13. Sperm with unstable chromatin structure (%) in diluted, Percoll-treated and oviduct-bound sperm. a,b,c: significantly different (P < 0.05) within a boar.

4.4 Prevalence of chromatin instability in accessory sperm

4.4.1 Morphological evaluation of sperm used for artificial insemination

The diluted semen of all three boars showed similar motility and percentages of sperm with morphological abnormalities other than cytoplasmic droplets. The semen from Boar 7 showed significantly lower (P < 0.05) percentages of sperm with unstable chromatin, cytoplasmic droplets and total abnormalities than the semen from Boars 5 and 6. These characteristics were similar in the semen from Boars 5 and 6 (Table 5).
Table 5. Motility, morphology and chromatin instability of sperm used for artificial insemination. Sperm was evaluated after 24 h of storage. a,b: indicate significant differences among the boars (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Boar 5</th>
<th>Boar 6</th>
<th>Boar 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculates (n)</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Gilts inseminated</td>
<td>4</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Sperm with unstable chromatin (%)</td>
<td>7.2±2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.8±9.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1±1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>85.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.0±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.8±8.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total sperm with morphological abnormalities (%)</td>
<td>36.0±2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3±2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8±1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Morphological abnormalities, other than cytoplasmic droplets (%)</td>
<td>8.0±2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5±3.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytoplasmic droplets (%)</td>
<td>28.0±4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.8±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3±2.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

4.4.2 Effect of semen storage on chromatin instability and on sperm morphology

The evaluation of four ejaculates from the control boar (Boar 7) showed a significantly higher (P < 0.05) percentage of sperm with unstable chromatin after 7 days of storage (from 1.5 to 35.9% from day 1 to day 7). This boar also had a significantly lower (P < 0.01) percentage of motility (from 73.8 to 2.8% from day 1 to day 7). The results of the morphological evaluation of this boar were significantly different (P < 0.05) only in respect to the acrosome defects (from 3 to 15% from day 1 to day 7). In Boar 6, all parameters remained similar during storage (n = 4 ejaculates; Table 6).
Table 6. Effect of long-time storage (7 days) on sperm quality parameters. n = 4 samples per boar; evaluation of the same sample at both storage intervals. a,b: indicate significant differences among the storage lengths within a boar (P < 0.01). c,d: indicate significant differences among the storage lengths within a boar (P < 0.05). 1Includes acrosome defects.

<table>
<thead>
<tr>
<th></th>
<th>Boar 6</th>
<th></th>
<th>Boar 7</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>7 days</td>
<td>1 day</td>
<td>7 days</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>82.5±2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.8±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.8±4.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8±4.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sperm with unstable chromatin (%)</td>
<td>4.7±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5±0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.9±0.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acrosome defects (%)</td>
<td>1.8±1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8±1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.8±4.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

4.4.3 Number of gilts inseminated; embryos and accessory sperm evaluated

The gilts used in this experiment had natural reproductive cycles and spontaneous ovulation. Although semen was available every day of the week, not all boars were available every day. Two shipments of semen were received per week, though semen each boar was normally received only once a week. As a result, the number of gilts inseminated per boar varied, such that semen from Boars 5 and 7 was used for 4 gilts each and semen from Boar 6 for 6 gilts; a total of 14 gilts were inseminated.

36 embryos and oocytes sired by Boar 5, 81 embryos and oocytes from Boar 6 and 54 embryos and oocytes from Boar 7 were evaluated. This made a total of 171 embryos and oocytes evaluated.

As explained in the materials and methods section, 500 sperm cells were counted in diluted semen. In the case of accessory sperm, all sperm cells in a given slide were counted when fewer than 500 sperm cells were found. When the number of sperm cells in a given slide was high (>500), ca. 500 cells were counted. At least one slide was counted per gilt and in some cases up to four (mean = 2.6 slides per gilt). As a result, the mean number of accessory sperm counted per gilt was 2129 sperm cells for gilts inseminated with semen from Boar 5; 1215 for Boar 6 and 1009 for Boar 7. An overall mean of 1417 sperm cells were counted per gilt.
4.4.4 Percentage of sperm with unstable chromatin in diluted semen and in accessory sperm

In the boars that had more than 5% sperm with unstable chromatin, the percentage of chromatin instability was lower (P < 0.05) in accessory sperm than in diluted sperm (6.5% in diluted semen and 1.9% in accessory sperm in Boar 5; 8.9 in diluted sperm and 1.4% in accessory sperm in Boar 6). In the control boar (Boar 7) no significant difference was observed (2.1% in diluted sperm and 1.0% in accessory sperm). The boar did not exert an effect on the percentage of chromatin instability in the accessory sperm (Figure 14).

The percentage of sperm with unstable chromatin correlated significantly with the percentage of cytoplasmic droplets (r = 0.7, P < 0.01) and of total abnormalities (r = 0.85, P < 0.01).
Figure 14. Percentage of sperm with unstable chromatin in inseminated semen and in accessory sperm of day 3-5 embryos. a,b: Significant differences within a boar (P < 0.05). n = 4 gilts (Boars 5 and 7); 6 gilts (Boar 6).

4.4.5 Effect of the embryo morphology on the percentage of chromatin instability in accessory sperm

The quality of the embryo (i.e. normal vs. degenerated) did not exert an effect on the percentage of chromatin instability in accessory sperm (Table 7).

<table>
<thead>
<tr>
<th>Type of embryo</th>
<th>Embryos (n)</th>
<th>Accessory sperm with unstable chromatin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>115</td>
<td>1.4±0.7</td>
</tr>
<tr>
<td>Degenerated</td>
<td>44</td>
<td>1.3±0.8</td>
</tr>
</tbody>
</table>

Table 7. Effect of the type of embryo on the percentage of accessory sperm with unstable chromatin.
4.4.6 Effect of the boar on fertilization

The boar did not exert an effect on the fertilization results (Table 8).

<table>
<thead>
<tr>
<th>Boar</th>
<th>Gilts (n)</th>
<th>Sperm with unstable chromatin (%)</th>
<th>Embryos and oocytes (n)</th>
<th>Fertilization Rate (%)</th>
<th>Rate of normal embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4</td>
<td>6.5±2.4</td>
<td>36</td>
<td>74.2±39.5</td>
<td>40.0±45.5</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>8.9±8.8</td>
<td>81</td>
<td>100.0±0.0</td>
<td>84.5±22.5</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>2.1±1.1</td>
<td>54</td>
<td>100.0±0.0</td>
<td>59.7±29.0</td>
</tr>
</tbody>
</table>

Table 8. Effect of the boar on fertilization rate and rate of normal embryos. A Percentage of sperm with unstable chromatin in diluted semen after 24 h of storage.

4.5 Accessory sperm: a bio-monitor of boar fertilizing capacity in vivo

4.5.1 Animals excluded

The insemination to ovulation interval for each sow was determined retrospectively. Twelve sows, whose insemination to ovulation interval was higher than 24 h, were excluded from the experiment. This was done because in vivo aging of sperm, which is caused by long insemination to ovulation intervals, has been found to lower both the accessory sperm counts and the percentage of normal embryos (WABERSKI et al., 1994a; ARDON et al., 2003).

4.5.2 Semen quality

Boar 8 had acceptable (5.2%) levels of cytoplasmic droplets (distal and proximal), while Boars 9 and 10 showed levels that were slightly elevated (18.7 and 20.1, respectively) (WABERSKI et al., 1994). The length of storage did not affect sperm motility, viability, or the percentage of total abnormal forms; all these factors were similar for all boars.
4.5.3 Effect on the number of oocytes ovulated on litter type, percentage of normal embryos and accessory sperm

The number of oocytes ovulated (estimated through the corpora lutea found in the ovaries) was not significantly different among the litter types (11.7, 10.9, 12.7, 11.9 and 11.4 for litters 1, 2, 3, 4 and 5, respectively). The number of corpora lutea per sow had no effect on any parameter studied.

4.5.4 Relationship between litter type and both accessory sperm count and percentage of normal embryos

The accessory sperm count in normal embryos from type 1 litters was significantly higher ($P < 0.01$) than in normal embryos from type 2 litters. In turn, the accessory sperm count in normal embryos from type 2 litters was significantly higher ($P < 0.01$) than that in type 3 litters. Accessory sperm counts were higher in normal embryos from type 3 litters than in those from type 4 litters (Table 9). The frequency of each zona class between litter types was also significantly different ($P < 0.01$) in all cases, except between type 3 and 4, which were similar in this respect. Because of the manner in which litter types were classified, it was expected that the percentages of normal embryos would be different in the different litter types. Type 1 litters had a significantly higher ($P < 0.01$) percentage of normal embryos than the other litter types, while type 2 and 3 litters showed similar percentages of normal embryos, which were significantly higher ($P < 0.01$) in these litter types than in type 4 litters (Table 9). The percentage of normal embryos found in type 4 litters was significantly higher ($P < 0.01$) than that of type 5 litters.
Table 9. Relationship between litter type and accessory sperm count in normal embryos.

<table>
<thead>
<tr>
<th>Litter type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litters (n)</td>
<td>34</td>
<td>17</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Corpora lutea (n)</td>
<td>398</td>
<td>185</td>
<td>89</td>
<td>166</td>
</tr>
<tr>
<td>Oocytes (n)</td>
<td>0</td>
<td>0</td>
<td>21</td>
<td>50</td>
</tr>
<tr>
<td>Degenerated embryos (n)</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Normal embryos (n)</td>
<td>398</td>
<td>143</td>
<td>68</td>
<td>75</td>
</tr>
<tr>
<td>Median percentage of normal embryos (%)</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Median accessory sperm in normal embryos</td>
<td>44.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Embryos per zona class&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7&lt;sup&gt;g&lt;/sup&gt;</td>
<td>19&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 (%)</td>
<td>12</td>
<td>28</td>
<td>54</td>
<td>45</td>
</tr>
<tr>
<td>3 (%)</td>
<td>28</td>
<td>27</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>4 (%)</td>
<td>58</td>
<td>38</td>
<td>18</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 9. Relationship between litter type and accessory sperm count in normal embryos.  

a,b,c,d: rows differ P < 0.01.  e,f,g: significant differences (P < 0.01) in the distribution of the zona classes.  

1Litter type 1 contained only normal embryos; type 2, normal and degenerated embryos; type 3, normal embryos and oocytes; type 4, oocytes and both normal and degenerated embryos.  

2Zona class 1: embryos that had 0-1 accessory sperm; class 2: 2-10 accessory sperm; class 3: 11-29 accessory sperm; class 4: 30 or more accessory sperm.

4.5.5 Relationship between embryo quality and accessory sperm count

Overall, embryos classified as normal had significantly higher (P < 0.05) accessory sperm counts than embryos classified as degenerated (median = 24.0 and 6.0, respectively; Figure 15). The distribution of the zona classes was also significantly (P < 0.01) different. In normal embryos, the proportion of zonae with more than 30 accessory sperm was higher than in degenerated embryos (45.0% and 6.0%, respectively), and the proportion of zonae with 0-1 accessory sperm was lower in normal than in degenerated embryos (6% and 24%, respectively). When analyzing the embryos within a litter type, it was found that, in type 2 litters, the accessory sperm counts were significantly higher (P < 0.05) in normal embryos than in degenerated embryos (median = 19.0 and 13.0, respectively; Figure 14). The zona class distribution was similar for both groups: 38% of normal embryos and 29% of degenerated embryos had more than 30 accessory sperm, while 8% of normal embryos and
14% of degenerated embryos had 0-1 accessory sperm. Accessory sperm counts were similar in normal and degenerated embryos from type 4 litters (median = 6.0 and 4.0, respectively) (Figure 15). The zona class distribution was also significantly different: there was a higher proportion of zonae with more than 30 accessory sperm in normal than in degenerated embryos (15.0% and 2.0%, respectively). There also were lower proportions of zonae with 0-1 accessory sperm in normal than in degenerated embryos (19% and 34%, respectively). The overall correlation between accessory sperm count and percentage of normal embryos was weakly significant (r = 0.42, P < 0.01).

**4.5.6 Effect of the boar on accessory sperm count, percentage of normal embryos, distribution of zona classes and type of litter**

The boar had a significant effect on the accessory sperm count, with Boar 8 having a significantly higher (P < 0.01) accessory sperm count than Boars 9 and 10; in turn, Boar 9 had a significantly higher (P < 0.01) accessory sperm count than Boar 10 (median = 53.0, 27.0 and 8.0 for Boars 8, 9 and 10, respectively). The overall frequency of zona classes per boar was significantly different in the three boars (P < 0.01). There were more than 30 accessory sperm in 60% of the normal embryos sired by Boar 8, in 48% of those sired by Boar 9 and in 13% of those sired by Boar 10. At the same time, only 0-1 accessory sperm was found in 4% of the normal embryos sired by Boar 8, in 4% of those sired by Boar 9 and in 11% of those sired by Boar 10. Although the percentage of normal embryos was similar for all boars (median = 100%, 84.6% and 80.1%, respectively), the proportion of type 1 litters was higher for Boar 8 than for Boars 9 and 10 (52%, 34% and 35%, respectively). The overall distribution of litter types for Boar 8 was significantly different (P < 0.05) than that of Boars 9 and 10, which were also similar to each other in this respect. Accessory sperm count and percentage of normal embryos correlated significantly for Boar 9 (r = 0.45; P < 0.05) and marginally significantly for Boar 10 (r = 0.49; p = 0.053); no significant correlation was found for Boar 8. Figure 16 shows the percentage of normal embryos and accessory sperm count per sow and by boar.
Figure 15. Relationship between embryo quality and accessory sperm\(^1\). a,b: significant differences between normal and degenerated embryos (P < 0.05). \(^1\)All: all litter types. Litter type 2 contained normal and degenerated embryos, litter type 4 contained oocytes plus normal and degenerated embryos.

Figure 16. Median percentage of normal embryos and median accessory sperm count per sow, according to the boar.
6. Discussion

The goal of semen quality assessment is facilitating sperm fertility prognosis. The concept of what is “good” or “bad” semen quality changes as new technologies emerge and more parameters can be evaluated. Traditional evaluation parameters include semen volume, color, odor, as well as sperm concentration, morphology and motility. Modern technology has allowed for the evaluation of new functional and morphological characteristics of sperm.

The measurement of male fertility is a difficult task. Male fertility in domestic animals can be defined as the capacity of their sperm to establish pregnancies with adequate numbers of vital, developing, competent embryos in a large number of females. It depends on several factors, including the number of spermatozoa per dose, the quality of the ejaculate, the type of dilution media and the age of the semen (JOHNSON et al., 1988; WABERSKI et al., 1994a). The fertility observed in the field is the product not only of male but also of female fertility and many sources of variation, which might not all be determinable (AMANN and HAMMERSTEDT, 2002). Therefore, insemination trials under field conditions are often not appropriate to assess the fertilization capacity of sperm, especially when the effect of a specific sperm defect is to be studied. Semen evaluation should include tests for the capacity of the sperm to fertilize and to support embryonic development in vivo, as well as determine whether and to what extent sperm with specific defects are able to pass the various barriers in the female tract and reach and fertilize the oocyte. This requires the identification of sperm parameters that are crucial for the fertilization process and knowledge of the selection mechanisms. The selection of sperm in the reproductive tract has the function of ensuring that only a few, fertilizing-capable sperm cells have access to the oocyte (HARRISON, 2000; TALEVI and GUALTIERI, 2004; HOLT and VAN LOOK, 2004).

A morphological characteristic that has been the focus of recent studies is the chromatin structure stability. A number of studies in various species indicate that chromatin instability is related to subfertility (bull: BALLACHEY et al., 1987; JANUSKAUSKAS et al., 2003; KHALIL, 2004. Mice: AHMADI and NG, 1999. Human: LIU and BAKER, 1992; SPANO et al., 2000; BUNGUM et al., 2004). To date, however, the mechanisms and the extent of this relationship are still unclear, particularly in boars, where very little information about the relevance of chromatin instability is available (WABERSKI et al., 2002; VOLKER, 2004).
Traditionally, chromatin instability has been considered as a so-called “uncompensable defect”. According to SAACKE et al. (2000), sperm with these kinds of defects are not culled by the female reproductive tract, thus allowing said defective sperm to fertilize the oocyte; such a fertilization would ultimately lead to early embryonic death. Because of the absence of selective barriers for these traits, increasing the sperm number in the insemination dose will not diminish their detrimental effect on fertility (SAACKE et al., 1998; EVENSON et al., 2000). In contrast, sperm with abnormalities belonging to the “compensable defects” class are those that are culled by the reproductive tract, e.g. lack of motility and tail morphological abnormalities. Sperm that show these defects will not reach or interact with the oocyte. In this case, using a higher number of spermatozoa per dose can reduce the detrimental effect they have on fertility (SAACKE et al, 1998; AMANN and HAMMERSTEDT, 2002). To determine how, and to what extent, a given sperm defect affects fertility, it must be determined whether that defect is compensable or uncompensable.

The first goal of the present work was the evaluation of the prevalence and constancy of chromatin instability in a population of 173 boars from an artificial insemination (AI) station. The relationship of chromatin instability to standard sperm parameters was also assessed.

In contrast to standard sperm parameters, no threshold values have been defined for chromatin instability in most mammalian species, except for humans, where a threshold value of 30% chromatin instability in a semen sample was set for “significant lack of fertility potential.” Samples with 15-30% sperm with unstable chromatin are classified as having a “reasonable lack of fertility potential” and only samples with <15% are considered as potentially fertile (EVENSON et al., 1999). In farm animals, however, the reproductive goal is to obtain high fertility in a large number of females using preserved semen for insemination in vivo. Therefore, it is assumed that the threshold value indicating reduced fertility would be lower in farm animals than in humans. Based on the experiences gained in previous studies (WABERSKI et al., 2002; LÖHMER, 2003; VOLKER 2004) and on the fact that the threshold value for morphological head abnormalities is 5%, the threshold for chromatin instability was also set at 5%. The aim of this study was not to relate chromatin stability to fertilization results, which means that further studies with larger numbers of boars are necessary before a threshold value to select semen samples can be defined. In the present study, 14.5% of the boars had at least one semen sample with >5% chromatin instability. Such results in a given ejaculate, however, do not seem to signal chronic problems in the donor
boar: about half of the boars, whose semen had more than 5% of sperm with unstable chromatin, showed this problem in only one out of three consecutive ejaculates evaluated. Few boars (7 out of 173 tested) consistently had semen samples with more than 5% of chromatin-unstable sperm. The percentage of chromatin-unstable sperm per ejaculate varied from 0 to 22%. The results of this experiment concur with those of VOLKER (2004), where 10% of the boars showed more than 5% of chromatin-unstable sperm, only 31% of that 10% showed the problem in a subsequent ejaculate, and the percentage of chromatin-unstable sperm per ejaculate varied from 0.1 to 19%. These results also concur with the findings of WABERSKI et al. (2002), where the percentage of chromatin instability ranged from 0.6 to 17% and where 64% of the boars had less than 5% of unstable chromatin in their ejaculates. The percentages of chromatin instability in species less intensively selected for fertility than boars seem to be higher than in boars or bulls: up to 88% in humans (SPANO et al., 2000) and a mean value of 36% in subfertile stallions (KENNEY et al., 1995). However, in a boar with an acute febrile infection, more than 50% of chromatin-unstable sperm were seen (Waberski and Helms, personal communication).

In this experiment, chromatin instability was significantly correlated to the percentages of cytoplasmic droplets, which is a common morphological abnormality observed in boar semen. In a previous study, it was seen that when the percentage of cytoplasmic droplets in a given boar was extremely high (>50%), the chromatin instability was in all cases higher than 5% (VOLKER, 2004). Another study (WABERSKI et al., 2002), however, found no correlation between chromatin instability and other parameters. In a study of bull semen, although no correlation was found between chromatin instability and morphology, the most commonly occurring sperm abnormality found was that of cytoplasmic droplets (BALLACHEY et al., 1987). In humans, studies have found correlations between chromatin instability and percentage of cytoplasmic droplets as well as sperm morphology, motility, and concentration (IBRAHIM and PEDERSEN 1988; LIU and BAKER, 1992; MOLINA et al., 2001; FISCHER et al., 2003). The global results of these studies show that there is no constant correlation between chromatin instability and other sperm defects in farm animals. The inconsistency of a correlation between chromatin instability and other defects confirms that chromatin instability is a relatively independent parameter and that an evaluation specifically for this trait needs to be performed, as concluded in other studies (SPANO 2000; LÖHMER, 2003). Additionally, this same inconsistency led to the general assumption that no major selection for chromatin stability occurs at the otherwise very efficient barrier of the utero-
tubal junction. Recent studies have shown that, once sperm have entered the oviducts, sperm binding to the oviductal epithelia represents a further mechanism for the selection of vital sperm (SUAREZ et al., 1991; ELLINGTON et al., 1999; GUALTIERI and TALEVI 2003). Sperm binding to the epithelia in the lower oviductal isthmus is the basic mechanism of sperm reservoir formation in most mammalian species (SUAREZ, 2002). Relevant parts of this mechanism can be studied through \textit{in vitro} binding assays, though those tests lack the influence of the functional regulation of the local environment \textit{in vivo} and of the physical properties of oviductal secretions (HUNTER et al., 1998). In the present study, the oviduct explant assay (OEA) described by PETRUNKINA et al., (2001) was used, though with slight modifications. The OEA is closer, both morphologically and functionally, to the conditions \textit{in vivo} than cultured oviductal monolayers assays.

Using boar semen with average sperm quality, it was found that the binding index (BI) of oviductal explant-bound sperm changes with time. An increase in the number of sperm bound to the oviducts is seen at the beginning of the coincubation period (0-30 min), followed by a plateau (30-90 min) and then a slow decrease. Such a slow, progressive detachment from the oviductal explants concurs with results and conclusions from previous studies (\textit{in vitro}: TALEVI and GUALTIERI, 2004; \textit{in vivo}: MBURU et al., 1996, 1997). Sperm were coincubated with the oviductal explants in a medium (TALP) that has capacitating effects. Because of this, the variation in the number of bound sperm is most likely the result of two independent phenomena: the capacities of the sperm to bind to the oviduct and to respond to the induction of capacitation, the latter of which causes the sperm release from the oviduct (FAZELI et al., 1999). The number of sperm bound to the oviductal explants varied depending on the boar and on the semen age at all intervals measured. Sperm stored for longer periods of time (72 vs. 24 h) appeared to have a lower capacity to bind to the oviduct: in all the intervals measured, the mean number of sperm bound was significantly lower in sperm stored for 72 h. Additionally, the sperm started to be released from the oviduct more rapidly. Sperm stored for 24 h showed a maximum level of binding capacity between 30 and 90 min. In sperm stored for 72 h, that maximum level was reached at 30 min and descended significantly at 60 min. Based on the results of this experiment, a coincubation period of 30 min was set for further experiments. This assay was modified to maximize the number of sperm bound to the oviduct and to release the sperm in a rapid manner without affecting its chromatin structure.
The release of sperm from the oviduct proved to be a difficult task. The results of this study suggest that the release of porcine sperm from the oviduct is very complex and that there is no “magic” substance that, when used individually, will efficiently release the sperm from the oviduct in vitro. Previous studies have focused on the inhibition of binding and have demonstrated the involvement of a number of substances (GREEN et al., 2001; BUREAU et al., 2002; TÖPFER-PETERSEN et al., 2002). Once the sperm are bound to the oviduct, however, the oviductal cells appear to protect them from disturbing agents in a very effective manner. Even capacitation-inducing agents like calcium ionophore and proteolytic agents like pronase did not release a great percentage of sperm. This differs from the situation in the bovine, where heparin facilitates the release of sperm in a very efficient fashion (TALEVI and GUALTIERI, 2001). The differences between species could be caused by differences in the biochemical or molecular characteristics of the sperm-oviduct interaction.

The release of virtually all sperm was needed to achieve the goals of the present study. It is not known whether the release of sperm, i.e. the capacity of sperm to react to “releasing” agents is yet another selection mechanism. Theoretically, if a substance were found to facilitate the release of a majority of the sperm, it would be impossible to know whether these sperm had a different quality than those that remained bound. For this reason, the release of sperm was accomplished through snap-freezing in liquid nitrogen. This is a very drastic method, which damages the acrosome and plasma membrane of the sperm, thereby releasing all bound sperm from the oviduct. It has no effect, however, on the percentage of sperm with unstable chromatin. In fact, snap-freezing of semen samples in liquid nitrogen is a common method to preserve the chromatin status until the SCSA is performed (EVENSON et al., 1994; VOLKER, 2004).

Other modifications to the OEA were that oviductal “strips” (i.e., long pieces of oviductal epithelia; see 3.4.3) were used instead of oviductal explants and that more sperm were coincubated with the epithelia. This increased the number of sperm that could bind to the oviduct and be used for later recovery and evaluation. The modifications to the OEA allowed the evaluation of the chromatin structure of released sperm. A method to release sperm in an effective and rapid manner is still needed, especially if the parameter to be measured is affected by snap-freezing and when oviduct-bound sperm are wanted for the evaluation of fertilization. This would facilitate a more comprehensive study of the characteristics of sperm bound to the oviduct and of whether these sperm features are associated with higher
fertilization competence (TALEVI and GUALTIERI, 2004). The search for substances that enable the release of sperm in vitro may be more successful in the future, as understanding of the physiological mechanisms of sperm release from the oviduct epithelium in pigs grows.

It was observed that Percoll density gradient centrifugation lowers the numbers of sperm with unstable chromatin. This concurs with the results of KOSOWER et al. (1992), CLAASSENS et al. (1998), SAKKAS et al. (2000) and TOMLINSON et al. (2001). Percoll gradient centrifugation is a common treatment of sperm used for assisted reproduction techniques (GRANT et al., 1994), and for physiological studies. In the present study, Percoll centrifugation was used for removal of seminal plasma, extender and amorphous material (GRANT et al., 1994). It is believed that Percoll centrifugation might select sperm populations with higher motility (GRANT et al., 1994) and superior morphology (TOMLINSON et al., 2001) than the original semen sample. This selection also results in decreased prevalence of chromatin instability, indicating an association between different sperm parameters (KOSOWER et al., 1992; CLAASSENS et al., 1998; SAKKAS et al., 2000).

A major finding in this study was that sperm bound to and released from the oviducts in vitro had lower percentages of chromatin instability than in the semen sample co-incubated with the oviductal strips. This concurs with the observations of FISCHER et al. (2003) and of LÖHMER (2003), where in vitro binding to human and bull oviductal epithelia, respectively, was preferential for sperm with stable chromatin. These results suggest that chromatin instability could be closely related to plasma membrane changes that hinder the binding of sperm to the oviduct. A lower capacity to bind to the oviduct would lower the number of sperm found in the reservoir, which would likely reduce the fertilization chances.

Although the binding of sperm to the oviduct in vitro proved to be preferential for sperm with stable chromatin, it was possible that the situation in vivo was different. Like other in vitro assays, the OEA (and its modification) lacks the hormonal, biochemical and anatomical environment of the oviduct in vivo. Extrapolation of the results of in vitro studies to the in vivo situation should be done very cautiously (WABERSKI et al., 2005). An in vivo study, therefore, was conducted to determine the prevalence of chromatin instability in the fertilizing-competent sperm population.
The \textit{in vivo} model used in this study is based on the accessory sperm found in the zona pellucida of day 3-5 embryos. These are sperm, other than the one that fertilized the oocyte, that are allowed by the partial zona block in pigs to penetrate and become entrapped in the zona pellucida. In pigs, this may happen for more than 24 h, as long as viable, fertilizing capable sperm are present in the oviduct. The accessory sperm have successfully passed the uterus and the UTJ, have reached the oviducts, possess the ability to recognize, bind to and penetrate the zona pellucida; thus, they represent the highly selected population of fertilizing competent sperm in the oviduct (HUNTER, 1984; HOLT and VAN LOOK, 2004). The aim of this part of the study was to compare the incidence of chromatin instability in accessory sperm to that of the sperm used for insemination.

In boars with elevated amounts of chromatin-unstable sperm, lower percentages (<3%) of those sperm were found in the accessory sperm population. Because the chromatin is enclosed within the sperm head, a direct communication between chromatin and the female tract is unlikely, which suggests that chromatin stability is associated with other sperm attributes that are part of the selection criteria in the female tract. Some could be parameters like sperm motility, morphology and cytoplasmic droplets: both these parameters and chromatin instability are affected by Percoll treatment. An association with these parameters would prevent the entrance of sperm with unstable chromatin into the ovaries \textit{in vivo} (BALLACHEY et al., 1988; IBRAHIM and PEDERSEN, 1988; LIU and BAKER, 1992; FISCHER et al., 2003; VOLKER, 2004). From the results of the oviduct explant assay, it can be concluded that sperm chromatin is also associated with changes in the plasma membrane, which hinder sperm binding to the oviductal epithelia. This assumption is supported by recent studies in this laboratory performed using bull sperm, where a relationship was found between chromatin stability and the ability of the sperm to regulate their cell volume in different osmotic media, which is a functional parameter of plasma membrane integrity. Another study in bulls also found a correlation between chromatin instability and disturbances of the plasma membrane function, as detected by the staining of sperm with annexin-V (JANUSKAUSKAS et al., 2003).

It is important to consider that chromatin instability in boar sperm has not shown a constant correlation with any one sperm defect. Sperm that are normal in all morphological and functional aspects, except for the stability of their chromatin structure, could avoid being rejected by the reproductive tract. Such sperm can interact with the oocyte \textit{in vivo}, as was
observed in the present study in <3% of the accessory sperm. It is assumed that this type of sperm would be able to fertilize the oocyte, but would not be able to support embryonic development. Some studies have correlated early embryonic death to sperm chromatin deficiencies (bull: BALLACHEY et al., 1987; JANUSKAUSKAS et al., 2003; KHALIL, 2004. Mice: AHMADI and NG, 1999. Human: LIU and BAKER, 1992; SPANO et al., 2000; BUNGUM et al., 2004). Under in vitro fertilization conditions, the barriers of the female tract are bypassed, thus increasing the chance that chromatin-unstable sperm will come into contact with the oocyte. Those sperm are likely to penetrate the oocyte in human assisted reproduction techniques (HOSHI et al., 1996; AHMADI and NG, 1999). However, it is not clear to what extent chromatin-unstable sperm are able to penetrate the zona pellucida in vitro. Theoretically, the zona pellucida could act as a barrier to chromatin-associated sperm deficiencies, something that would be bypassed were ICSI used instead of IVF. However, it would be extremely difficult to set adequate conditions that would allow us to track the fate of isolated, chromatin-unstable sperm throughout the process of fertilization, making absolute clarification of these suggestions unlikely.

The main difference between the present trial and the Hannover Gilt Model, described by ARDON et al. (2003), is that in the present experiment, gilts were inseminated several times using commercial sperm doses (containing 2 x 10⁹ sperm) instead of once with a dose containing low sperm numbers (i.e. 0.8 x 10⁹ sperm, as used in the last experiment of this study). Such low numbers of sperm would have been needed if the focus of the present study had been the evaluation of the fertilization capacity of semen samples with high percentages of chromatin instability. Instead, high numbers of sperm needed to be inseminated to provide sufficient accessory sperm to obtain reliable data about the percentages of sperm with unstable chromatin that are found in the zona pellucida. As a result, 19,838 accessory sperm from 159 embryos were considered in this analysis. Interestingly, the percentage of accessory sperm with unstable chromatin was low in all embryos and did not differ between normal and degenerated embryos, meaning that no effect of the embryo quality on the prevalence of chromatin instability was found.

For the final experiment, the focus shifted from qualitative to quantitative evaluation of accessory sperm. The goal was to determine whether accessory sperm counts are a valid parameter for the evaluation of semen quality in vivo and was done using the Hannover Gilt Model. As stated above, this model is characterized by the use of single insemination with
low sperm numbers and the monitoring of ovulation time. The use of low numbers of sperm in the insemination dose allows for the evaluation of semen samples based on the differences in the fertilization chances caused by compensable defects, whose effect would otherwise be reduced, thereby remaining undetected, when the sperm numbers in the dose are high (AMANN and HAMMERSTEDT, 2002). As stated above, the number of accessory sperm in the zona pellucida of porcine embryos reflects the number of fertilization competent sperm in the oviduct. The goals of this experiment were to determine whether it was possible for the quality of the embryo’s zona pellucida to affect the number of accessory sperm, regardless of the number of sperm in the oviduct (as DEJARNETTE et al. (1992) theorized was the situation in bovines), and whether the quantitative evaluation of accessory sperm would provide information about semen quality in addition to that obtained through the determination of the fertilization rate and the rate of normal embryos. To clarify these points, the relationship between the accessory sperm counts, embryo quality and the origin of the embryo was evaluated.

In this experiment, the embryos were divided into different litter types according to their quality. Different litter types reflect different levels of fertilization success. The different levels of fertilization success are reflected in the accessory sperm count in normal embryos, where the highest count was found in litters containing only normal embryos and the lowest in litters comprised of both normal and degenerated embryos, as well as oocytes. To assure that differences between groups were not caused by a small number of embryos with extremely high accessory sperm counts (i.e., up to 200), all embryos with more than 30 accessory sperm were grouped into one class. Even then, differences among groups were apparent.

To determine whether the oocyte quality influences the accessory sperm count, regardless of the number of sperm present in the oviduct, the type of litter from which the embryos came was taken into account. In previous studies, embryos were treated as independent entities, without analysis of the effect due to factors affecting whole litters (DEJARNETTE et al., 1992; WABERSKI et al., 1994a; SOEDE et al., 1995; ARRIOLA and FOOTE, 2001). This is particularly true of studies performed in cattle (DEJARNETTE et al., 1992), as each embryo is equivalent to a litter. In the present study, an apparent relationship between embryo quality and accessory sperm was found when the traditional approach was applied. Under further analysis, however, it was observed that the litter, and not the quality of the individual embryo,
was the main source of that relationship, although it cannot be proven that the oocyte quality had no effect. The results of this study show that the litter type has an effect on the accessory sperm count and that the effect of embryo quality is not constant. Normal embryos had significantly more accessory sperm than degenerated embryos in litters containing only normal and degenerated embryos; however, in litters where oocytes were also present the accessory sperm count was low in both normal and degenerated embryos.

The lack of a constant effect of the embryo quality is also evident in the fact that normal embryos can have very low accessory sperm numbers (only 0-1 in 6%). This is true even in 1.2% of normal embryos from litters consisting entirely of normal embryos. Degenerated embryos, on the other hand, can have very high accessory sperm counts: one such embryo had 200 accessory sperm, while 11 embryos (16%) had more than 30. Oocytes, on the other hand, had very low accessory sperm counts in all litters, usually (in 82% of the cases) no more than one (0-1). The findings that the accessory sperm count is consistently unaffected by embryo quality and that it correlates weakly to the percentage of normal embryos indicate that accessory sperm are an independent variable. The information provided by the accessory sperm cannot be gained either through embryo quality or the percentage of normal embryos. Thus, the information obtained from the accessory sperm count is not redundant. Additionally, accessory sperm count may indicate differences in the fertilization capacity of semen samples even before this is expressed in different percentages of normal embryos. It is speculated that this is the situation observed with the boar effect. The percentages of normal embryos do tend to differ, although not significantly, and the superiority of a given boar can be perceived to be significant through the accessory sperm counts. This concurs with the results of both the distribution of litter types, which differed significantly between the boars, and the boars’ in vitro semen evaluation. The in vitro semen evaluation showed that Boar 8 had fewer sperm cells with cytoplasmic droplets than boars 9 and 10. Cytoplasmic droplets are believed to be a primary defect, originating in the testes from a membrane defect that hinders physiological migration of the cytoplasmic droplet in the epididymis (LARSSON et al., 1980). It has been recommended that the total percentage of cytoplasmic droplets in an ejaculate should not exceed 15%, especially when stored semen is used (WABERSKI et al., 1994b). Boars 9 and 10 exceeded this limit; their lower fertilization potential was shown in the accessory sperm count found in the normal embryos they sired. Similarly, type 2 litters had significantly higher accessory sperm counts than type 3 litters, but the percentages of normal embryos remained similar. It is theorized that type 2 litters (consisting of normal and
degenerated embryos) are superior to type 3 litters (consisting of normal embryos and oocytes); that is, fertilization conditions are assumed to have been better in the case of gilts showing embryo degeneration than in gilts showing partial fertilization. Although both litter types show the same percentage of normal embryos, accessory sperm numbers differ. This suggests that accessory sperm counts are a more sensitive parameter of sperm quality than the rate of normal embryos. The results of this study show that the accessory sperm counts are affected by the embryo’s litter type and not by the quality of the individual embryo. Additionally, it was found that accessory sperm counts do not repeat information obtained with either embryo quality or percentage of normal embryos. Accessory sperm counts are a sensitive indicator of the number of fertilization-competent sperm present in the oviduct. It is suggested that accessory sperm counts offer additional, valuable information that improves the semen evaluation capabilities of in vivo models, such as the Hanover Gilt Model.

In summary, from the series of experiments presented in this thesis, it can be concluded that chromatin instability in boar sperm is associated with plasma membrane changes, which hinder the binding of chromatin-unstable sperm to the oviductal epithelia, thereby reducing their number in the functional sperm reservoir of the female reproductive tract. Association with other sperm defects might also hinder the entrance of chromatin-unstable sperm into the oviducts. This questions the traditional view that chromatin-disturbed sperm would not be selected in the female tract and that, consequently, their chances to fertilize would only depend on their prevalence in the ejaculate. The results of this study suggest that, as the sperm traverse the female reproductive tract, the percentage of chromatin instability is reduced through the culling of sperm with deficiencies associated to chromatin-instability. Therefore, chromatin-instability could be regarded as more of a “compensable defect”, because the enhancement of the sperm number in the insemination dose should, in theory, reduce the negative effect of this defect on fertility in pigs. The present study, however, was not aimed at determining the actual impact of chromatin instability on boar fertility. To evaluate effectively the fertilization capacity of semen, based on chromatin instability, it would be advisable to use the Hannover Gilt Model, which uses fertilization rate, the rate of normal embryos and accessory sperm counts as tools to evaluate semen quality. In this study, accessory sperm counts were proven a valuable tool for the evaluation of the fertilization capacity of semen samples.
6 Summary

Florence Ardón Martínez

Studies on the interaction of chromatin-unstable boar sperm with the female reproductive tract

The aim of this study was to elucidate the relevance of sperm chromatin instability for the fertilization capacity of boar semen. This was achieved using *in vitro* and *in vivo* models to analyze the interaction of chromatin unstable sperm with the female reproductive tract.

Chromatin instability is generally believed to be one of the main causes of embryonic mortality and it is presumed that there is no specific selection for chromatin stability in the female reproductive tract. Thus, sperm with unstable chromatin structure might be able to fertilize the oocyte, but not to sustain embryonic development, thereby causing early embryonic death.

In this study, the semen of 173 boars from a commercial boar stud was evaluated. It was found that a relatively low percentage (14.5%) of them had high rates of chromatin instability in their ejaculates and that few of them (4%) showed this problem chronically. Chromatin instability was correlated significantly (r=0.44, P=0.01) with the percentage of sperm with plasma droplets. Two studies were done to determine whether chromatin stability is part of the selection criteria in the reproductive tract. First, the chromatin structure of diluted boar sperm was compared to that of Percoll®-treated sperm and of sperm released from oviductal epithelia *in vitro*. Sperm were treated with Percoll® gradient centrifugation to eliminate seminal plasma and extender. Then, Percoll®-treated sperm (n=14 ejaculates from four boars) were co-incubated with oviductal epithelia (n = 2 sows per boar) for 30 min in a 5% CO₂, 39 °C, atmosphere. Sperm were released from the oviduct through snap-freezing in liquid nitrogen. Chromatin stability was evaluated using the modified fluorescent microscopic Sperm Chromatin Structure Assay (mFSCSA). Sperm released from the oviduct had significantly fewer (P<0.05) sperm with unstable chromatin than Percoll®-treated or diluted sperm. Percoll®-treated sperm showed significantly fewer (P<0.05) sperm with unstable chromatin in two boars.
A second experiment was performed to determine whether, and to what extent, sperm with unstable chromatin could reach and penetrate the zona pellucida of oocytes *in vivo*. Accessory sperm are those found in the zona pellucida, thus considered competent for fertilization. The diluted semen of two donor boars had elevated percentages of sperm with unstable chromatin (more than 5%); a third boar was used as a control. 14 gilts were inseminated up to three times with $2 \times 10^9$ sperm diluted in BTS. The gilts were evaluated every 12 h to determine estrus and ovulation. 3 to 5 days after ovulation, the gilts were slaughtered and their reproductive tracts flushed for embryo recovery. The morphology of the embryos was evaluated under a stereomicroscope. The chromatin stability of the sperm used for insemination and of the accessory sperm found in the zona pellucida of the embryos was evaluated using the mfSCSA. In both boars with more than 5% chromatin instability, accessory sperm had significantly lower (P<0.05) percentages of chromatin instability than the sperm that were used for insemination.

A further goal of this study was to assess the value of quantitative analysis of accessory sperm for use in the study of the effect of chromatin disturbance and other sperm defects on fertilization capacity. 96 gilts were each inseminated with max. 30 h stored semen from one of three boars. Ovulation was detected in 12 h intervals using transcutaneous sonography. Embryos were recovered 3 to 5 d after ovulation. Neither the artificial insemination to ovulation interval nor the boar exerted an effect on the fertilization rates of normal embryos. The boar had a significant effect on accessory sperm numbers. There was a significant relationship between accessory sperm count and litter quality. Embryos from litters with a 100% fertilization rate (normal embryos only) had significantly higher accessory sperm counts than normal embryos from litters where either embryonic degeneration or partial fertilization was also present.

The results of these studies show that sperm with stable chromatin are more likely both to bind to the oviduct *in vitro* and to traverse the reproductive tract *in vivo*, ultimately reaching the oocytes and penetrating the zona pellucida. This might be caused by the association of chromatin instability with plasma membrane changes, which prevent the binding of the sperm to the oviductal epithelia. Additionally, chromatin instability might be related to other defects, which might hinder the passage of chromatin-unstable sperm into the oviducts. Based on these results, it is suggested that sperm with unstable chromatin have lower probabilities of fertilizing oocytes than chromatin-stable sperm. This study, however, was not intended to
determine the actual impact of chromatin instability on boar fertility. To evaluate effectively the fertilization capacity of semen, based on chromatin instability, it would be advisable to use the Hannover Gilt Model, which uses fertilization rate, the rate of normal embryos and accessory sperm counts as tools to evaluate semen quality. In this study, accessory sperm counts were proven a valuable tool for the evaluation of fertilization capacity of semen samples.
7 Zusammenfassung

Florenzia Ardón Martínez  
**Untersuchungen zur Interaktion von chromatin-instabilen Eberspermien mit dem weiblichen Genitaltrakt**


Chromatin-Instabilität wird allgemein als eine der Hauptursachen für embryonale Mortalität angesehen und es wird angenommen, dass keine spezifischen Selektionsmechanismen für Chromatininstabilität im weiblichen Genitale existieren. Somit könnte ein Spermium mit instabiler Chromatinstruktur die Eizelle zwar befruchten, aber nicht die embryonale Entwicklung aufrechterhalten, wodurch es zum Tod des Embryos käme.

In dieser Studie wurde Sperma von 173 Ebern einer kommerziellen Besamungsstation untersucht. Dabei konnte festgestellt werden, dass ein relativ geringer Prozentsatz (14,5 %) der Eber hohe Werte an Chromatin-Instabilität aufwiesen und dass einige davon (4 %) dieses Problem chronisch zeigten. Chromatin-Instabilität war signifikant korreliert (r=0,44; P=0,01) mit dem Prozentsatz an Plasmatropfen. Es wurden zwei Studien durchgeführt, um zu analysieren, ob die Chromatin-Stabilität Teil der Selektionskriterien im weiblichen Geschlechtstrakt ist. Zuerst wurde die Chromatinstruktur von verdünnten Eberspermien mit der von Percoll-behandelten Spermien und von oviduktgebundenen Spermien *in vitro* verglichen. Die Spermien wurden durch Percoll-Gradienten-Zentrifugation von Seminalplasma und Verdünner befreit. Dann wurden die Percoll-behandelten Spermien (n=14 Ejakulate von 4 Ebern) für 30 min in einem 39 °C warmen 5 % CO2-haltigen Milieu mit Eileiterepithel (n=2 Sauen pro Eber) inkubiert. Die Spermien wurden durch Schockgefrierung in flüssigem Stickstoff vom Eileiter abgelöst. Dann wurde die Chromatininstabilität mittels modifiziertem floureszensmikroskopischem Spermienchromatinstruktur-Assay (mfSCSA) untersucht. Bei den vom Eileiter freigesetzten Spermien gab es signifikant weniger (P<0.05) chromatin-instabile Spermien als beim Percoll-behandelten oder verdünnten Sperma. Percoll-
behandeltes Sperma von zwei Ebern wies signifikant weniger (P<0.05) Spermien mit instabilem Chromatin auf.

Es wurde eine zweite Studie durchgeführt, um zu untersuchen, ob und bis zu welchem Grad die chromatin-unstabilen Spermien die Zona pellucida der Eizellen in vivo erreichen und durchdringen können. Akzessorische Spermien sind diejenigen, die in der Zona pellucida gefunden werden und somit als befruchtungskompetent angesehen werden. Der verdünnte Samen von zwei Ebern wies erhöhte Prozentzahlen an Spermien mit instabilem Chromatin auf (mehr als 5 %); ein dritter Eber diente als Kontrolle. 14 Jungsauen wurden bis zu dreimal mit 2 Milliarden Spermien, verdünnt in BTS, besamt. Die Jungsauen wurden alle 12 Stunden untersucht, um Östrusbeginn und Ovulationszeitpunkt zu bestimmen. Drei bis fünf Tage nach der Ovulation wurden die Jungsauen geschlachtet und der Genitaltrakt zur Embryonengewinnung gespült. Die Morphologie der Embryonen wurde unter dem Stereomikroskop untersucht. Die Chromatinstabilität der Spermien, die für die Besamung verwendet wurden und die der akzessorischen Spermien, die sich in der Zona pellucida befanden, wurde mittels mfSCSA analysiert. Bei den beiden Ebern mit mehr als 5 % Chromatin-Instabilität zeigten die akzessorischen Spermien signifikant geringere (p<0,05) Prozentsätze an Chromatin-Instabilität als das für die Besamung verwendete Sperma.

8 References


9 Appendix

9.1 Equipment

9.1.1 Semen collection
- Collection dummy
- Semen collection vessel
- “USbag” collection bags
- Semen collection gloves
- Hygienic glove (to protect the collection glove)

9.1.2 Artificial insemination
- 100 ml boar semen bottle with cap
- “Spirette” artificial insemination catheter for pigs

9.1.3 Ovulation time detection
- 5 mHz Sector Probe Ultrasound “Sonoline” SI 250

9.1.4 Semen evaluation, preparation and dilution
- Microprocessor pH/IONmeter PMX 3000
- Magnetic stirrer with heating stage (MR3001 K)
- 10 - 40 x binocular phase-contrast microscope
- Phase-contrast binocular microscope
- Waterbath with temperature regulation (0-100 °C)
- Temperature-controlled semen storage unit
- Duran® Measuring cylinders, low form with hexagonal base (100, 250 and 500 ml)
- Eppendorf precision pipettes (100-1000 ml; 10-100 µl)
- Pipette tips for Eppendorf pipette
- Microscope Slide (76 x 26 mm)
- Microscope Slidecover (18 x 18 mm) Interessengemeinschaft der Laborfachhändler, Nidderau
- Thoma neu counting chamber Brand, Wertheim
- Safe-Lock micro test tubes (1 ml) Eppendorf, Hamburg
- Plastic reagent and centrifuge tubes Sarstedt, Nümbrecht
  with screw cap (10 ml)

9.1.5 Embryo evaluation

- Contrast-phase fluorescent microscope (Axioskop 40) Carl Zeiss, Oberkochen
  with Ploem illuminator
- Warming plate (470 x 363 cm) Minitüb, Tiefenbach
- Stereo Microscope with 6x to 40x magnification Wild Heerbrugg, Hamburg
- Laboratory centrifuge (megafuge 2.0 R) Heraeus Instruments, Hamburg
- Duran® beakers low form with graduation and Shott Geräte, Mainz
  spout (150 ml)
- Duran® centrifuge tubes with conical Shott Geräte, Mainz
  bottom (30°, 10 ml)
- Dissecting scissors (Aesculap, 16.5 cm, Braun medical, Seesatz,
  blunt-sharp points) Switzerland.
- Bowel forceps Hauptner Instrumente, Dietlikon,
  Switzerland
- Curved haemostat Hauptner Instrumente, Dietlikon,
  Switzerland
- 50 ml Syringes B. Braun medical, Seesatz,
  Switzerland
- Knobbed needle Hauptner Instrumente, Dietlikon,
  Switzerland
- Plastic petri dishes (5 and 3.5 cm diameter) Greiner Bio-One, Solingen-Wald
- Plastic reagent and centrifuge tubes with Sarstedt, Nümbrecht
  screw cap (50 ml)
- Safe-Lock micro test tubes (1 ml) Eppendorf, Hamburg
- Micro-pipette and attachments Band, Wertheim
- 20µl Capillary pipettes “Unopette” Becton Dickinson, Franklin Lakes,
- Microscope Slide (76 x 26 mm) Interessengemeinschaft der Laborfachhändler, Nidderau
- Microscope Slidecover (18 x 18 mm) Interessengemeinschaft der Laborfachhändler, Nidderau
- Single-use syringe 20 ml B Braun, Melsungen, Melsungen
- Sterilizing filter unit Millex®-GP (35 mm) Millipore, Bedford, MA, USA

9.1.6 Oviduct Explant Assay (OEA) / Oviductal strips sperm binding and release
- Research Stereo Microscope SZX12 (7x to 90x) Olympus, Hamburg
- Inverse Microscope IM35 Carl Zeiss, Oberkochen
- Video Camera CF 8/1 Kappa opto-electronics, Monrovia, CA, USA
- Video Monitor WV-BM 1400 Panasonic, Secaucus, NJ, USA
- Video-recorder SLV-E 720, VHS Sony, New York, NY, USA
- CO2-Auto-Zero incubator Heraeus Instruments, Hamburg.
- Warming plate micro stage (HT 200) Minitüb, Tiefenbach.
- AIDA software (Bildanalyse Ver. 2.0, Copyright 1992) Milka Medical, Rosenheim.
- Duran® Laboratory bottles, round shape, graduated, with screw cap (100 and 500 ml) Shott Geräte, Mainz.
- Operating Scissors (Aesculap, 16.5 cm, blunt-sharp points) Braun medical, Seesatz, Switzerland
- Iris scissors (Aesculap, 9.5 cm, sharp-sharp points) Braun medical, Seesatz, Switzerland
- Medium Dissecting Forceps (straight, 1.15cm) Braun medical, Seesatz, Switzerland
- Dumont micro-surgery forceps (45°, 11 cm) Fine Science tools, Heidelberg
- Extra delicate mini-vannas iris spring scissors (straight, sharp-sharp points, 7.5 cms; 3 mm cutting edge) Fine Science tools, Heidelberg
- Hypodermic needles Sterican® (1.2 x 50 mm) B Braun Melsungen, Melsungen
- Duran® Glass petri dish (5 cm diameter) Shott Geräte, Mainz
- Plastic petri dish (5 and 3.5 cm diameter) Greiner Bio-One, Solingen-Wald
- Plastic culture dish with 4 wells (3.5 cm diameter) Greiner Bio-One, Solingen-Wald
- Plastic reagent and centrifuge tubes with screw cap  
  (10 and 50 ml)  
  Sarstedt, Nümbrecht
- Safe-Lock micro test tubes (1 ml)  
  Eppendorf, Hamburg
- Microscope Slide (26 x 76 mm)  
  Interessengemeinschaft der Laborfachhändler, Nidderau
- Microscope Slidecovers (24 x 60 mm).  
  Interessengemeinschaft der Laborfachhändler, Nidderau
- Paraffin wax for histology  
  Merck, Darmstadt
- Silicone Stopcock Grease  
  Dow Corning, Midland, MI, USA

9.1.7 Modified fluorescent Sperm Chromatin Structure Assay (mfSCSA)
- Analytical laboratory scale  
  Sartorius, Göttingen
- Fluorescent microscope with contrast-phase and difference-interference-contrast device  
  Zeiss (axioskop), Jena
- Microscope digital camera  
  (Olympus DP 50 for Windows)  
  Olympus, Hamburg
- analySIS® 3.0 software  
  Soft Imaging System, Münster
- Superfrost® Plus slide (25 x 75 x 1 mm)  
  Menzel-Gläser, Braunschweig
- Duran® beaker low form with graduation and spout (10 and 50 ml)  
  Shott Geräte, Mainz
- Duran® Measuring cylinders, low form with hexagonal base (100, 250, 500 and 1000 ml)  
  Shott Geräte, Mainz
- Duran® Laboratory bottle, round shape, dark colored, graduated, with screw cap (100 and 1000 ml)  
  Shott Geräte, Mainz
- Duran® Laboratory bottle, round shape, graduated, with screw cap (100 and 500 ml)  
  Shott Geräte, Mainz
- Hellendahl vertical glass stain jar, with glass lid to fit  
  Carl Roth, Karlsruhe
- Duran® glass test tube (10 ml, rounded bottom)  
  Shott Geräte, Mainz
- Wash bottles polythene oval (500 ml)  
  Carl Roth, Karlsruhe
- Glass mohr pipette with 1 ml graduations (20 and 10 ml)  
  Brand, Wertheim
- Glass mohr pipette with 0.1 ml graduations (5 ml)  
  Brand, Wertheim
- Microcentrifuge tubes with screw cap  
  Sarstedt, Nümbrecht
- Parafilm M® sealing film  
  American National Can, Menasha, WI, USA
9.2 Chemicals and reagents

- Absorption tube for H₂O Merck, Darmstadt

- Acetone (CH₃COCH₃, M=58.08) Merck, Darmstadt

- Acetic Acid (C₂H₄O₂, M=60.05, 100% DAB) Carl Roth, Karlsruhe

- Acridine Orange (C₁₇H₂₀ClN₃, M=301.82) Merck, Darmstadt

- Bovine Serum Albumin (BSA) Fraction V Merck, Darmstadt

- Calcium Chloride (CaCl₂ M=111.0, pure, 96%, anhydrous) Serva Electrophoresis, Heidelberg

- Calcium Chloride Dehydrate (CaCl₂ x 2H₂O, M=147.01, extra pure food grade) Merck, Darmstadt

- Chloroform (CHCl₃, M=119.32, extra pure) Merck, Darmstadt

- Citric Acid Monohydrate (C₆H₈O₇ x H₂O, M=210.14) Carl Roth, Karlsruhe

- 1,4-Dithiothreitol (DTT; C₄H₁₀O₂S₂, M=154.2) Carl Roth, Karlsruhe

- Ethanol (C₂H₅OH, M=46.07 Rotipuran ≥ 99.8% RG) Merck, Darmstadt

- Ethylenedinitrilo Tetraacetic Acid, Disodium Salt Dehydrate (Tritriplex III, EDTA; C₁₀H₁₄N₂Na₂O₈ x 2H₂O, M=372.24) Merck, Darmstadt

- Formaldehyde (CH₂O, M=30.03, ≥ 35% DAB) Carl Roth, Karlsruhe

- D (-) Fructose (C₆H₁₂O₆, M=180.16, for microbiology) Merck, Darmstadt

- Gentamicin Sulfate 708 U/mg, RG Serva Electrophoresis, Heidelberg

- D (+) Glucose Monohydrate (C₆H₁₂O₆ x H₂O, M=198.17, for biochemistry) Merck, Darmstadt

- Hepes (C₈H₁₈N₂O₄S, M=238.31, buffer grade) Applichem, Darmstadt

- Hoechst Dye 33342 Sygma, Deisenhofen

- Lactic Acid (C₃H₅NaO₃, M=112.07) Merck, Darmstadt

- Magnesium Chloride Anhydrous (MgCl₂, M=95.2) Aldrich-Chemie, Steinheim

- Magnesium Chloride Hexahydrate (MgCl₂ x 6H₂O, M=203.3, GR) Merck, Darmstadt

- D(+)-Mannose (C₆H₁₂O₆, M=180.16) Merck, Darmstadt

- Methanol 79% (CH₃OH, M=32.04, RG) Carl Roth, Karlsruhe

- Dimethylsulfoxide Anhydrous (DMSO; (CH₃)₂SO, ≥99.5 %, RG) Carl Roth, Karlsruhe
9.3 Solutions

9.3.1 Solutions for sperm evaluation, preparation and dilution

**Sodium chloride solution for counting of sperm, 10%**

- Sodium Chloride 10 g
- Distilled Water (ad) 100 ml
Sperm fixing solution for morphology assessment (modified from HANCOCK, 1956)
- Formaldehyde solution: 37 ml formaldehyde in 100 ml water
- Tri-Sodium Citrate solution: 2.9 g of Tri-Sodium Citrate Dehydrate e in 100 ml water.
- Fixing solution: 4 ml of formaldehyde solution in 96 ml of Tri-Sodium Citrate solution.

Propidium iodide (PI) staining solution

1. Tyrode’s Salt Solution
   - Sodium Chloride 5.844 g
   - Potassium Chloride 0.2 g
   - Sodium Bicarbonate 0.84 g
   - Sodium Dihydrogen Phosphate Anhydrous 0.048
   - Lactic Acid 2.802 g
   - Magnesium Chloride Anhydrous 0.047 g
   - Hapes 6.0 g
   - D (+) Glucose Monohydrate 1.087 g*
   - Pyruvic Acid Sodium Salt 10 ml *
   - Bovine Serum Albumine 6.0 g *
   - Distilled water (ad) 1 L

* Added the day of use.

The osmolarity has to be ca. 300 mOsm/Kg. The pH was set to 7.4. This solution was frozen in 10-ml plastic centrifuge tubes. It must be thawed 2 days before use.

2. PI base solution

2 mg PI in 1 ml of Tyrode’s Salt Solution.

3. SYBR base solution

1 mg SYBR 14 in 1 ml DMSO.
Percoll\textsuperscript{TM} Solution

1. Percoll\textsuperscript{TM} Base solution

- Sodium Chloride \hspace{1cm} 8 g
- Heps \hspace{1cm} 4.77 g
- D (+) Glucose Monohydrate \hspace{1cm} 1.98 g
- Potassium Hydroxide \hspace{1cm} 2.5 ml of a 1M base solution

First, the chemicals were placed in a 100-ml graduated cylinder. Next, the cylinder was filled up to 70 ml with distilled water. The pH was set at 7.4. The cylinder was then filled up to 100 ml with distilled water and the pH was set at 7.6. It was divided into 10-ml plastic centrifuge tubes and frozen. Of these tubes, two were needed to prepare the 35% and 70% Percoll\textsuperscript{TM} solutions.

2. M Solution:

10.8 of the Percoll\textsuperscript{TM} base solution were sterilized by filtration and placed in a 100-ml graduated cylinder. Distilled water was added up to 100 ml. Next, 250 µl of Gentamicin solution (at 20 mg/ml) were added. The pH was set to 7.4 and osmolarity should be ca. 295 mOsm/Kg. The osmolarity value corresponds to “m” in the formulas.

3. 1 + 9 Solution:

5.4 g of the Percoll\textsuperscript{TM} base solution were sterilized by filtration and placed in a 100-ml graduated cylinder. Next, 50.9 g of undiluted Percoll\textsuperscript{TM} (ca. 45 ml) were added. Osmolarity was measured. It should be ca. 350 mOsm/Kg. The osmolarity value corresponds to “dp” in the formulas.

4. Osmolarity measurements:

The osmolarity from Percoll\textsuperscript{TM} was measured; it should be ca. 15 mOsm/Kg. This value corresponds to “p” in the formulas.

After all osmolarity values were obtained, the following values were estimated.

\begin{align*}
O_a &= 0.1 \left(10m + 9p\right) = \text{ca. } 322 \\
R &= \frac{O_a - 0.1dp}{0.9dp} = \text{ca. } 0.86 \\
V_p &= \frac{10m - 295}{R \left(295 - p\right)} = \text{ca. } 11
\end{align*}
5. Percoll™ 100% Solution

\[ V_0 = (V_p - 9) (1.13) \]  

The \( V_0 \) of undiluted Percoll™ was added to the 1+9 solution by filtration. Osmolarity was measured and should be ca. 295 mOsm/Kg. This solution will be divided into two and each will correspond to \( V_1 \) for the next formulas.

6. Percoll™ 70% Solution

\[ V_2 = V_1 \times \frac{100}{70} \]

Half of the Percoll™ 100% Solution (ca. 33 ml) was placed in a graduated cylinder and was filled with M solution up to \( V_2 \). Osmolarity should be ca. 300 mOsm/Kg.

7. Percoll™ 35% Solution

\[ V_3 = V_1 \times \frac{100}{35} \]

Half of the Percoll™ 100% Solution (ca. 33 ml) was placed in a graduated cylinder and was filled with M solution up to \( V_3 \). Osmolarity should be ca. 300 mOsm/Kg.

The Percoll™ solutions (35 and 70%) were placed at 4°C and can be used for about two weeks.

**Beltsville Thawing Solution** (BTS; JOHNSON et al., 1988)

- D (+) Glucose Monohydrate 37 g
- Tri-sodium Citrate dehydrate 6 g
- Sodium Bicarbonate 1.3 g
- EDTA 1.3 g
- Potassium Chloride 0.8 g
- Gentamicin Sulfate 0.25 g
- Distilled water (ad) 1 L

The solution was set at pH 6.8 y 280-320 mOsm/kg.
**Androhep (WEITZE et al., 1990)**

- D (+) Glucose Monohydrate 13 g
- Tri-Sodium Citrate Dehydrate 4 g
- Sodium Bicarbonate 0.6 g
- EDTA 1.2 g
- Bovine Serum Albumin 1.25 g
- Hepes 4.75 g
- Gentamicin sulfate 0.3 g
- Distilled water (ad) 500 ml

The solution was set at pH 6.8 and 280-320 mOsm/kg.

**Androhep without EDTA**

- D (+) Glucose Monohydrate 7.8 g
- Tri-Sodium Citrate Dehydrate 2.4 g
- Sodium Bicarbonate 0.36 g
- Hepes 2.7 g
- Gentamicin Sulfate 0.12
- Distilled water (ad) 300 ml

The solution was set at pH 7.4 and 280-320 mOsm/kg. It was frozen in 10-ml tubes. The day of use, 0.025 g of Bovine Serum Albumin were added to the solution.

**9.3.2 Solutions for the evaluation of embryos produced in vivo**

**PBS (phosphate buffered solution) for flushing of the oviducts**

- Sodium Chloride 8g
- di-Sodium Hydrogen Phosphate Dehydrate 1.26 g
- Potassium Dihydrogen Phosphate Anhydrous 0.2 g
- Potassium Chloride 0.2 g
- Distilled water, ad 1 L

The PBS was frozen in 50-ml plastic tubes with screw cap. The day of use, 0.02 g of Bovine Serum Albumin was added to 100 ml of PBS. 40 ml were used for the flushing of each of the oviducts.
uterine horns. From the rest of the PBS, about 10 ml each were poured into small petri dish (5 ml per dish) and the beakers were rinsed with the remaining liquid.

**Hoechst staining solution:**
- Base solution 1. 1 mg Hoechst dye 33342 per 2 ml distilled water
- Base solution 2. 100µl Base solution 1 per 1 ml distilled water
- Final concentration: 50µg Hoechst 33242 per ml distilled water

The staining solution was stored in 1-ml Eppendorf tubes at –20 °C and protected from light with aluminum foil. The stain had to be warmed up to room temperature before staining. Because of the adverse effects of the temperature changes, the stain in a given tube was only used a maximum of 3 times.

**Siliconizing solution:**
- Ethanol
- Acetone
- Silicone Oil solution (2 ml of Silicone Oil and 98 ml of Chloroform).

To siliconize the beakers, they were washed thoroughly, then rinsed one time with acetone and twice with alcohol. After each rinsing, the beakers were air-dried. Finally, all were filled with the silicone oil solution and left for three minutes so that the silicon could adhere to the glass. Afterwards, they were air-dried for one hour and then sterilized for either 2 hours at 240-260 °C or 4 hours at 200 °C.

**9.3.3 Solutions for the Oviduct Explant Assay (OEA) / Oviductal strips sperm binding and release**

**Phosphate Buffered Saline (PBS)**
- Sodium Chloride 90 g
- di-Sodium Hydrogen Phosphate Dehydrate 16.2 g
- Potassium Dihydrogen Phosphate Anhydrous 3.4 g
- Distilled water (ad) 1 L
This was the base solution, which was frozen in 50-ml plastic centrifuge tubes. The work solution was prepared by thawing 50 ml of the base solution and diluting it 1.10. The pH was set to 7.4 and the osmolarity to 280-320 mOsm/Kg. For each work day, 100 ml were sterilized by filtration and 50 mg streptomycin and 75 mg penicillin added to it.

**Tyrode-Albumin-Lactate-Pyruvate (TALP) medium (PARRISH et al, 1988)**

1. **base solutions**
   - Sodium Chloride 13.33 g
   - Potassium Chloride 1.176 g
   - Sodium Bicarbonate 1.260 g
   - Sodium Dihydrogen Phosphate Monohydrate 0.47 g
   - Calcium Chloride Dehydrate 2.940 g
   - Magnesium Chloride Hexahydrate 2.034 g

   The chemicals were diluted each in 100 ml distilled water; these solutions can be used for up to a week.
   - Gentamicin Sulfate 25 mg/ml of distilled water
   - Sodium Pyruvate 0.022 mg/10 ml of distilled water

   These base solutions were placed in 1-ml Eppendorf tubes and frozen.

2. **TALP final solution**
   - Sodium Chloride base solution 21.7 ml
   - Potassium Chloride base solution 9.8 ml
   - Sodium Bicarbonate base solution 50 ml
   - Sodium Dihydrogen Phosphate Monohydrate base solution 5 ml
   - Calcium Chloride Dehydrate base solution 5 ml
   - Magnesium Chloride Hexahydrate base solution 5.5 ml
   - Hapes 1.1 g
   - Sodium Lactate 1.2 g
   - Distilled water (ad) 500 ml
pH was set to 7.4, the solution sterilized by filtration and frozen in 50-ml portions.

Before its use, each portion of TALP was thawed and the following chemicals added to it:

- Gentamicin Sulfate base solution 500 µl
- Sodium Pyruvate base solution 55 µl
- Bovine Serum Albumin 300 mg

Final osmolarity should be between 280 and 320 mOsm/Kg.

**Heparin Solution for sperm release**

1. **Base solution**
   - TALP (or Tyrode) medium 50 ml
   - Bovine Serum Albumine 0.3 g
   - Pyruvic Acid Sodium Salt 5.5 g
   - Gentamicin Sulfate 2.5 mg

2. **Final solution**
   - Base solution 20 ml
   - Heparin 2 mg

The final solution has to be incubated for one hour in the CO₂ chamber before use.

**5% Pronase solution for sperm release**

1. **Base solution**
   - Sodium Chloride 0.9 mg
   - Bidistilled water 10 ml

2. **Final solution**
   - Base solution 10 ml
   - Pronase 5 mg

**Calcium ionophore solution for sperm release**

1, 5 and 10 µM each in 10 ml TALP medium
Mannose solution for sperm release

0.05 and 0.1 M each in TALP medium

9.3.4 Solutions for the mfSCSA (VOLKER, 2004)

Buffer solution
- Sodium Chloride 29 g
- EDTA 3.36 g
- Bidistilled water (ad) 1 L

pH was set to 6.8. The solution was placed in a dark colored Duran® glass laboratory bottle. This solution can be used for up to a month.

DTT/DMSO solution
- DTT 0.046 g
- DMSO 2.5 ml
- Bidistilled water 1485 µl

The DTT was placed in a 50 ml glass beaker. 30 ml of buffer solution were added to the DTT. The beaker was covered with Parafilm M®. Separately, ca. 3.5 ml DMSO were placed in a 20 ml glass beaker. Then, 1485 µl bidistilled water were placed in a glass test tube. 3195 µl DMSO were placed on the plastic test tube. This DMSO solution was left, covered with Parafilm M®, to react for ca. 5 minutes; then, it was mixed with the DTT. This solution should be prepared and left under an extractor. It can be used for up to 24 h.

Carnoy’s solution
- Methanol 40 ml
- Acetic acid 20 ml

The methanol and the acetic acid were placed into a Hellendahl vertical glass stain jar and mixed thoroughly. The jar should be completely covered with aluminum foil. This solution is prepared shortly before use.
Acridin-orange staining solution
- di-Sodium Hydrogen Phosphate Anhydrous solution 2.5 ml
- Citric Acid solution 40 ml
- Acridine Orange solution 10 ml

The solutions were placed in a Hellendahl vertical glass stain jar and mixed thoroughly. The jar should be cold and placed inside a cocktail mixer. This solution is prepared shortly before use.

di-Sodium Hydrogen Phosphate Anhydrous solution
- di-Sodium Hydrogen Phosphate Anhydrous 5.34 g
- Bidistilled water (ad) 100 ml

The solution was placed into a 100-ml laboratory bottle and refrigerated. This solution can be used for up to a month.

Citric Acid Monohydrate solution
- Citric Acid Monohydrate 10.05 g
- Bidistilled water (ad) 500 ml

The solution was placed into a 500-ml laboratory bottle and refrigerated. This solution can be used for up to a month.

Acridine orange solution
- Acridine orange 0.1 g
- Bidistilled water (ad) 100 ml

The solution was placed into a 100-ml dark colored laboratory bottle and refrigerated. It should be covered with aluminum foil. This solution can be used for up to a month.
Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die Dissertation:

Characterization of fertilizing competent boar spermatozoa in the female genital tract

Selbständig verfasst habe. Bei der Anfertigung wurden folgende Hilfen Dritter in Anspruch genommen:


2. Die Statistische Auswertung der Ergebnisse wurde nach Beratung mit und unter Anleitung von Dr. Martin Beyerbach (Institut für Biometrie, Epidemiologie und Informationsverarbeitung) unter Verwendung des Statistikprogramms SAS vorgenommen.


4. Die fachliche Beratung wurde von Prof. Dr. med. Vet Dagmar Waberski (Institut für Reproduktionsmedizin) übernommen.


Ich habe die Dissertation an folgenden Institutionen angefertigt:
Institut für Reproduktionsmedizin

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.
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