BINDING CAPACITY OF BULL SPERMATOZOA TO OVIDUCTAL EPITHELIUM IN VITRO AND ITS RELATION TO SPERM CHROMATIN STABILITY, SPERM VOLUME REGULATION AND FERTILITY

INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
DOKTORS DER VETERINÄRMEDIZIN
(Dr. med. vet.)
durch die Tierärztliche Hochschule Hannover

Vorgelegt von

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Date of the oral examination: 18.11.2004
TO MY FAMILY, especially my PARENTS
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<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>AO</td>
<td>Acidin orange</td>
</tr>
<tr>
<td>BI</td>
<td>Binding index</td>
</tr>
<tr>
<td>BOEC</td>
<td>Bovine oviductal epithelial cells</td>
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<tr>
<td>BR</td>
<td>Blastocyst rate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>°C</td>
<td>Grade Celsius</td>
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<tr>
<td>ca</td>
<td>Circa</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
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<tr>
<td>CASY1</td>
<td>Cell counter and analyzer system</td>
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<tr>
<td>Cm</td>
<td>Centimetre</td>
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<tr>
<td>CMA</td>
<td>Cell motion analyzer</td>
</tr>
<tr>
<td>CR</td>
<td>Cleavage rate</td>
</tr>
<tr>
<td>CTC</td>
<td>Chlortetracycline</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>1,4 Dithiotreit</td>
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<tr>
<td>D.W</td>
<td>Double distilled water</td>
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<tr>
<td>eCG</td>
<td>Equine chorionic gonadotropin</td>
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<tr>
<td>EDTA</td>
<td>Ethylene di-amine tetra acetic acid</td>
</tr>
<tr>
<td>EN</td>
<td>Eosin/Nigrosin</td>
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<tr>
<td>et al</td>
<td>et alii (and others)</td>
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<td>Explant</td>
<td>Part or section of living tissue which taken out from the natural site of growth and place in a medium for culture</td>
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<td>Gram</td>
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<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HCG</td>
<td>Human chronic gonadotropine</td>
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<td>Hepes</td>
<td>H-[2-Hydroxyethyl] piperazin-N’-[Ethansulfonic acid]</td>
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<td>HHE</td>
<td>Heparin, hypotaurin and epinephrin</td>
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<td>HOST</td>
<td>Hypo-osmotic swelling test</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
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<tr>
<td>IVC</td>
<td>In vitro culture</td>
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<td>IVF</td>
<td>In Vitro Fertilization</td>
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<td>IVM</td>
<td>In vitro maturation</td>
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<td>IVP</td>
<td>In vitro embryo production</td>
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<td>Kg</td>
<td>Kilogram</td>
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<td>M</td>
<td>Molar</td>
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<td>mf-SCSA</td>
<td>Modified fluorescence microscopical sperm chromatin structure assay</td>
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<td>mg</td>
<td>Milligram</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>µl</td>
<td>Micro litre</td>
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<td>Micrometer</td>
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<td>min</td>
<td>Minute</td>
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<td>Mio</td>
<td>Million</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mOsm/kg</td>
<td>Milliosmolal</td>
</tr>
<tr>
<td>mOsm/L</td>
<td>Milliosmolar</td>
</tr>
<tr>
<td>No</td>
<td>Number</td>
</tr>
<tr>
<td>OEA</td>
<td>Oviduct-explant-assay</td>
</tr>
<tr>
<td>OECE</td>
<td>Oviduct epithelial cells explants</td>
</tr>
<tr>
<td>OECM</td>
<td>Oviduct epithelial cell monolayers</td>
</tr>
<tr>
<td>p</td>
<td>Probability of the zero hypotheses</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PH</td>
<td>Hydrogen ion concentration</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl pyrrolidone</td>
</tr>
<tr>
<td>®</td>
<td>Registered trade mark</td>
</tr>
<tr>
<td>RVD</td>
<td>Regulative volume decrease of modal sperm volume</td>
</tr>
<tr>
<td>RVS</td>
<td>Relative volume shift (modal value)</td>
</tr>
<tr>
<td>SCSA</td>
<td>Sperm chromatin structure assay</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOF</td>
<td>Synthetic oviduct fluid</td>
</tr>
<tr>
<td>TALP</td>
<td>Tyrode, Albumin, Lactate, Pyruvate medium</td>
</tr>
<tr>
<td>TB</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>TCM</td>
<td>Tissue culture medium</td>
</tr>
<tr>
<td>TM</td>
<td>Total motile spermatozoa</td>
</tr>
<tr>
<td>UTJ</td>
<td>Utero-tubal junction</td>
</tr>
<tr>
<td>VAP</td>
<td>Average path velocity of spermatozoa (µm/sec.)</td>
</tr>
<tr>
<td>VCL</td>
<td>Curvilinear velocity of spermatozoa (µm/sec.)</td>
</tr>
<tr>
<td>VSL</td>
<td>Straight-line velocity of spermatozoa (µm/s)</td>
</tr>
<tr>
<td>Vi5m</td>
<td>Mean sperm volume under iso-osmotic conditions at 5 min</td>
</tr>
<tr>
<td>Vh5m</td>
<td>Mean sperm volume under hypo-osmotic condition at 5 min</td>
</tr>
<tr>
<td>Vi20m</td>
<td>Mean sperm volume under iso-osmotic condition at 20 min</td>
</tr>
<tr>
<td>Vh20m</td>
<td>Mean sperm volume under hypo-osmotic condition at 20 min</td>
</tr>
<tr>
<td>RVSm</td>
<td>Relative shift of mean sperm volume after 5 min</td>
</tr>
<tr>
<td>Vr20m</td>
<td>Relative shift of mean sperm volume after 20 min</td>
</tr>
<tr>
<td>RVDm</td>
<td>Regulative decrease of mean sperm volume</td>
</tr>
<tr>
<td>Vi5</td>
<td>Modal sperm volume under iso-osmotic conditions at 5 min</td>
</tr>
<tr>
<td>Vh5</td>
<td>Modal sperm volume under hypo-osmotic condition at 5 min</td>
</tr>
<tr>
<td>Vi20</td>
<td>Modal sperm volume under iso-osmotic condition at 20 min</td>
</tr>
<tr>
<td>Vh20</td>
<td>Modal sperm volume under hypo-osmotic condition at 20 min</td>
</tr>
<tr>
<td>Vr20</td>
<td>Relative shift of modal sperm volume after 20 min</td>
</tr>
<tr>
<td>RVS</td>
<td>Relative volume shift of modal values after 5 min</td>
</tr>
<tr>
<td>RVD</td>
<td>Regulative decrease of modal sperm volume</td>
</tr>
<tr>
<td>X</td>
<td>Arithmetic mean</td>
</tr>
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</table>
1 INTRODUCTION

In vivo several physiological barriers exist to ensure that spermatozoa that participate in fertilization are of the highest quality. Such barriers are thought to include penetration of the cervical mucus, transport through the female reproductive tract, storage and survival in the oviduct, penetration of cumulus cells and binding to the zona pellucida (HUNTER, 1995; BAILLIE et al., 1997). Specifically, sperm that reach the fallopian tube have a higher proportion of normal morphology than that seen for sperm lower in the tract (MORTIMER et al., 1982). At the time of insemination in many eutherian mammals millions of sperm are released to the reproductive tract, only thousands pass through the uterotubal junction into the oviductal isthmus, where they form a reservoir (HUNTER, 1998; LEFEBVRE et al., 1995). The sperm reservoir is created in various mammalian species such as cattle (HUNTER and WILMUT, 1984), rabbit (OVERSTREET and COOPER, 1978b), sheep (HUNTER and NICHOL, 1983), pigs (HUNTER, 1981), hamster (SMITH et al., 1987), and mice (SUAREZ, 1987). The sperm reservoir acts to ensure that enough fertile spermatozoa are available in the oviduct when ovulation occurs. Contact of the spermatozoa with the oviduct epithelial cells maintains viability of spermatozoa stored in the oviduct (SMITH and YANAGIMACHI, 1990) and prolongs their ability to fertilize (POLLARD et al., 1991; SMITH and YANAGIMACHI, 1991). As the time of ovulation nears, release of spermatozoa commences, and a few reaches the ampulla where fertilization takes place (HUNTER, 1998; SUAREZ et al., 1990). The interaction takes place between the sperm plasma membrane overlying the acrosome and the surface of the mucosal epithelium often via cilia (LEFEBVRE et al. 1995; SUAREZ, 1987; HUNTER et al., 1991; SMITH and YANAGIMACHI, 1991).

In the recent years, the interaction of spermatozoa with the oviduct epithelium has been studied using in vitro culture of spermatozoa and epithelial cells and more focused on the mechanisms and biological aspects of sperm oviduct binding. The following in vitro studies were conducted to further characterize functional aspects of the interaction between frozen-thawed bull spermatozoa and the oviductal epithelium. Differences between the initial binding capacity of frozen-thawed sperm to
oviductal epithelium using the oviductal explant assay (OEA) were studied in repeated ejaculates from 30 bulls. Additionally, the relationship between sperm-oviductal epithelium binding capacity and membrane functional integrity and chromatin stability, and also their relation to fertility in vitro were investigated.
2 REVIEW OF LITERATURES

2.1 THE OVIDUCTS AND SPERM STORAGE

2.1.1 Functional anatomy of the oviduct

The mammalian oviducts are anatomically divided into three sections, which in a caudal to cranial direction are: the isthmus, the ampulla and the infundibulum. The distinct cell types of the epithelium lining the oviductal isthmus are ciliated epithelial cells, which presumably facilitate gamete movement within the oviduct, and non-ciliated secretory cells, which are characterized by secretory granules and by microvilli on their apical surface. The morphology and ultrastructure of ciliated and secretory oviductal epithelial cells (OEC) have been described in large domestic animal species (cattle: STALHEIM et al., 1975; ABE and OIKAWA, 1993; BOLLO et al., 1990; goats: ABE et al., 1993; pigs: WU et al., 1976; horses: BOYLE et al., 1987; BADER, 1982; BALL, 1996) and changes in cell structure associated with the cycle stage of the female have been documented (ABE and OIKAWA, 1993; ABE et al., 1993). In the primate oviducts, cycles of ciliation and deciliation of the fimbria and ampulla have been reported, and reciliation appears to be controlled by circulating estrogen concentrations (BRENNER et al., 1974; ODOR et al., 1980). The epithelial height and activity of the secretory cells are increased around the time of ovulation (JANSEN, 1984; AMSO et al., 1994). The energy substrates as glucose, pyruvate and lactate originate from the plasma transudate through passive diffusion and the cells show glycolitic activity (LEESE, 1988; EDWARDS and LEESE, 1993), resulting in a low glucose concentration and some accumulation of lactate in the luminal fluid.

2.1.2 Sperm transport in the female genital tract

Sperm migration through the female tract is sustained by sperm swimming activity, muscular contraction of the tract and ciliary activity of the oviductal mucosa (BENNETT et al., 1988; AYAD at al., 1990; BARRATT and COOKE, 1991). Depending on the species and on the site of sperm deposition, by 1-8 hours after
insemination the fertilizing sperm will be found stored in the caudal isthmus. However uterine sperm may continue to enter the oviductal isthmus. Therefore, sperm cells may remain in the isthmus as long as 18-20 h in cattle, 17-18 h in sheep, and up to 36 h in pigs. Since most large farm animals will be mated at a specific phase of the estrous cycle, spermatozoa are present in the reproductive tract at or around the time of ovulation, establishing a temporal relationship between semen deposition and ovulation (HUNTER 1984; ORIHUELA et al., 1999). Moreover it was mentioned that sperm transport in to the oviducts involving adovarian waves of contraction and ciliary’s activity in the oviductal epithelium in rabbit (VANDEN-BOSCH and HAFEZ, 1974), pigs (BLANDAU and GADDUM-ROSSE, 1974), cattle (WILMUT and HUNTER, 1984), sheep (HUNTER and NICHOL, 1983) and humans (AHLGREN, 1975; BARRATT and COOKE, 1991). In horses the fertilizing population of spermatozoa reached the oviduct within two to four hours of insemination (BRINSKO et al., 1990; 1991), whereas this transport might take about eight hours in cattle (HAWK, 1987). Recently, an association between impaired sperm transport and subfertility has been suggested in horses (SCOTT et al., 1995).

2.1.3 Oviductal sperm reservoir

After natural mating, sperm are transported to the oviduct, where they form a reservoir. In species where sperm are deposited in the vagina, sperm enter the cervix and are stored in crypts from which they are subsequently released to reach the oviduct (HARPER, 1994). Evidence from studies in a variety of mammalian species supports the existence of sperm storage in the female oviduct prior to fertilization. In vivo studies characterizing oviductal sperm storage have been reported in mice (ZAMBONI, 1972; SUAREZ, 1987), rats (SHALGI and KRAICER, 1978), guinea pigs (YANAGIMACHI and MAHI, 1976), hamster (SMITH et al., 1987; SMITH and YANAGIMACHI, 1990; 1991), rabbits (OVERSTREET et al., 1978), sheep (HUNTER et al., 1980; 1982; HUNTER and NICHOL, 1983) pigs (HUNTER, 1981; HUNTER et al., 1987), cattle (HUNTER and WILMUT, 1984; LARSSON and LARSSON, 1986; HUNTER et al., 1991; LEFEBVRE et al., 1995), horses (PARKER et al., 1975; BADER, 1982), bats (RACY, 1975), marsupials (TAGGART and TEMPEL-
SMITH, 1991; BEDFORD and BREED, 1994) and humans (AHLGREN, 1975; MANSOUR et al., 1993; HUNTER, 1995). Evidence for oviductal sperm storage has also been derived from studies in vitro (cattle: ELLINGTON et al., 1991; POLLARD et al., 1991; Pigs: SUAREZ et al., 1991; horses: LEFEBVRE and SAMPER, 1993; ELLINGTON et al., 1993a; THOMAS et al., 1994a,b; humans: BONGSO et al., 1993; PACEY et al., 1995; MORALES et al., 1996). Prolonged storage of sperm in the female reproductive tract is also a well-described phenomenon in poultry (BAKST, 1994). In contrast, WILLIAMS et al. (1993) did not found evidence for an isthmic oviductal sperm reservoir in humans. It is noteworthy that while an oviductal sperm reservoir exists in marsupial mammals, the sperm do not appear to attach to the OEC (BEDFORD and BREED, 1994).

2.1.3.1 Formation of the oviductal-sperm reservoir

Several mechanisms have been proposed for the retention of spermatozoa in the oviductal isthmus. Spermatozoa could remain in the isthmus due to depressed sperm motility, as reduction of flagellar beat activity in spermatozoa in contact with the OEC has been reported (OVERSTREET and KATZ, 1977; SUAREZ, 1987; SUAREZ and OSMAN, 1987). Alternatively, it has been suggested that spermatozoa retained in the isthmic reservoir may be due to: localized constriction of the isthmus (OVERSTREET and COOPER, 1975; SUAREZ, 1987), attachment to the OEC (POLLARD et al., 1991; HUNTER, 1991), entrapment in locally secreted mucus (SUAREZ, 1987; SUAREZ et al., 1991), estrous stage related edema of oviductal mucosa (FLECHON and HUNTER, 1981; BOYLE et al., 1987) or a combination of these factors. Scanning electron microscopy of oviductal tissue recovered from bred mares and cows revealed spermatozoa in intimate contact with OEC (BADER, 1982; BOYLE et al., 1987; LEFEBVRE et al., 1995), and this was confirmed by studies using cocultures of spermatozoa and OEC from horses and cattle (ELLINGTON et al., 1991; 1993a; THOMAS et al., 1994a,b; SUZUKI and FOOTE, 1995). Some authors reported that spermatozoa attaching only to ciliated OEC (sheep: HUNTER and NICHOL, 1983; pigs: SUAREZ et al., 1991; PETRUNKINA et al., 2001b; cattle: POLLARD et al., 1991; LEFEBVRE et al., 1995; horses: ELLINGTON et al., 1993b),
whereas other studies revealed spermatozoa attaching to both ciliated and non-ciliated cells (hamsters: SMITH and YANAGIMACHI, 1990; cattle: HUNTER et al., 1991; SUZUKI and FOOTE, 1995; horses: BADER, 1982; THOMAS et al., 1994a). It is noteworthy that when bovine sperm placed into culture of fetal OEC bound only to the differentiated OEC that possessed cilia (POLLARD, 1992). On the other hand, sperm can bind to OEC monolayers that lack cilia (THOMAS et al., 1994b; GUTIERREZ et al., 1993). The storage of spermatozoa in oviductal reservoir appears to be physical attachment of spermatozoa to the OEC (mouse: SUAREZ, 1987; hamster: SMITH and YANAGIMACHI, 1989; 1990; rabbit: OVERSTREET and COOPER, 1978a,b; pig: HUNTER, 1981; 1984; FLECHON and HUNTER, 1981; SUAREZ et al., 1991; cattle: LARSSON and LARSSON, 1985; HUNTER et al., 1991; LEFEBVRE et al., 1995; horses: BADER, 1982). The interaction takes place between the sperm plasma membrane overlying the acrosome and the surface of the mucosal epithelium often via cilia (SUAREZ, 1987; SMITH and YANAGIMACHI, 1991; HUNTER et al., 1991; LEFEBVRE et al., 1995). Within the isthmic reservoir, spermatozoa attach to OEC by their rostral plasma membrane (HUNTER et al., 1987, 1991; POLLARD et al., 1991; SMITH and YANAGIMACHI, 1991; SUAREZ et al., 1991; ELLINGTON et al., 1993b; LEFEBVRE et al., 1995). Although the in vivo research in several species indicates that sperm reservoir is limited to the caudal isthmus (HUNTER, 1981; HUNTER and NICHOL, 1983; HUNTER, 1984; HUNTER and WILMUT, 1984; SMITH and YANAGIMACHI, 1990; HUNTER et al., 1991), the sperm binding with OEC in vitro is not limited to isthmic epithelium when sperm are given equal access to isthmic and ampullary epithelial cells. No anatomical regional effect on sperm binding in vitro was detected in pigs (SUAREZ et al., 1991; PETRUNKINA et al., 2001b) and cattle (NAGAI and MOOR, 1990; LEFEBVRE et al., 1995). However, in other studies an anatomical effect was observed in equine (THOMAS et al., 1994a) and human (BAILLIE et al., 1997). Comparatively little is known about the mechanisms of sperm binding to the OEC. Cell surface carbohydrates are important in cell adhesion (GAHMBERG et al., 1992) and play an important role in mammalian fertilization (AHUJA, 1985). Glycoprotein recognition has been implicated in the adhesion of spermatozoa to OEC. Glycoproteins
expressed on the luminal surface of OEC were characterized by lectin immunocytochemistry in mice (WHYTE et al., 1987), rabbits (MENGHI et al., 1985; 1995), pigs (RAYCHODHURY et al., 1993), horses (BALL et al., 1997) and humans (SCHULTE et al., 1985; WU et al., 1993). A lectin-like interaction between spermatozoa and OEC has been demonstrated, involving recognition of feutin and sialic acid in hamsters (DE MOTT et al., 1995), galactose in horses, (LEFEBVRE, 1997), fucose in cattle (LEFEBVRE and SUAREZ, 1996) and mannose in pigs (WAGNER et al., 2002). It has been reported that fucose blocks binding of bull sperm to bovine OEC in vitro (LEFEBVRE et al., 1997). Fucose-specific lectins have been used to demonstrate that fucose is densely distributed on the surface of bovine oviductal epithelium; furthermore, pre-treatment of OEC with fucosidase reduces sperm binding (LEFEBVRE et al., 1997). Specific recognition and binding between bovine sperm and homologous OEC are believed to be mediated by the interaction of a Ca$^{2+}$-dependant Lectins on the sperm head surface (DE MOTT et al., 1995; SUAREZ et al., 1998) and Fucose supported by a Lewis-A-trisaccharide present on the apical membrane of OEC (SUAREZ et al., 1998; LEFEBVRE et al., 1997; REVAH et al., 2000). Similar binding of bovine sperm to OEC has also been observed in vitro (ELLINGTON et al., 1991; POLLARD et al., 1991; CHIAN and SIRARD, 1995; SUZUKI and FOOTE, 1995; LEFEBVRE and SUAREZ, 1996; SUZUKI et al., 1997). This is evidence strongly suggests that fucose is involved in bovine sperm binding to oviductal epithelium.

2.1.3.2 Demonstration of the sperm oviduct binding

Sperm binding to OEC has been observed in all eutherian mammals in which the reservoir has been examined. Attachment of sperm to OEC has been demonstrated in vivo by Transillumination of excised whole oviduct in mice (SUAREZ, 1987) and hamster (KATZ and YANAGIMACHI, 1980). Scanning electron micrographs (SEM) of oviducts of mated pigs and cows indicate an apparent association between sperm and oviductal epithelium (SUAREZ et al., 1991; HUNTER at al., 1987; LEFEBVRE et al., 1995). In vitro sperm binding to oviductal explants has been used as a model for studying the sperm-oviduct interaction in several species including cattle.
ELLINGTON et al., 1991; POLLARD et al., 1991; LEFEBVRE et al., 1995), pigs
(SUAREZ et al., 1991; RAYCHOU DHURY and SUAREZ, 1991), horses (LEFEBVRE
and SAMPER, 1993; ELLINGTON et al., 1993a; THOMAS et al., 1994a,b) and
humans (BONGSO et al., 1993; PACEY et al., 1995; MORALES et al., 1996). Sperm
attachment to OEC monolayers has also been demonstrated in these species
(sheep: GUTIERREZ et al., 1993; equine: ELLINGTON et al., 1993b; bovine:
GUALTIERI and TALEVI, 2003).

2.2 FUNCTIONS OF THE OVIDUCTAL SPERM RESERVOIR

2.2.1 Sperm selection

In vivo several physiological barriers exist to ensure that sperm, which participate in
fertilization, are of the highest quality. Such barriers are thought to include penetration
of the cervical mucus, transport through the female reproductive tract, utero-tubal
junction (UTJ), storage in the fallopian tube, binding to and penetrate of cumulus cells
and the zona pellucida (HUNTER, 1995). Specifically, sperm that reach the fallopian
tube have a higher proportion of normal morphology than that seen for sperm lower
in the tract (MORTIMER et al., 1982), and sperm that bind to the zona pellucida have
more normal chromatin structure than those that do not (HOSHI et al., 1996). In
addition, co-culture of sperm with oviduct epithelial cells results in a stabilizing effect
for sperm against chromatin changes (ELLINGTON et al., 1998a). Establishment of
the oviductal sperm reservoir can serve to selectively retain uncapacitated,
morphologically normal sperm with intact acrosome from the population of
spermatozoa reaching the oviduct (MITCHELL et al., 1985; SHALGI et al., 1992;
THOMAS et al., 1994b; LEFEBVRE et al., 1995; PETRUNKINA et al., 2001b).
Furthermore, it was estimated that binding to oviductal cells is not only beneficial for
sperm survival but also represents a crucial step for the selection of spermatozoa
endowed with superior fertilization competence (GUALTIERI and TALEVI, 2003).
Sperm that did not attach to OEC during in vitro culture were the poorer quality sperm
in the sample. In particular, human sperm that did not attach had poorer motility and
lesser quality chromatin (ELLINGTON et al., 1999a). Recent studies suggest that an
intricate cooperation and synchrony exists between mammalian sperm and both OEC and oviductal secretion in the regulation of the events of reproduction including sperm survival and storage at the internal body temperature, sperm selection, capacitation and optimization of subsequent fertilization (BARRATT and COOKE, 1991; ZHU et al., 1994; KIM et al., 1996). Capacitated spermatozoa do not appear to attach to OEC (hamster: SMITH and YANAGIMACHI, 1990; 1991; stallion: THOMAS et al., 1995a; bull: LEFEBVRE and SUAREZ, 1996).

2.2.2 Maintaining viability, motility and fertilizing capacity of spermatozoa

Extended lifespan of spermatozoa and maintenance of sperm motility is a widely documented effect of sperm-oviduct interaction. *In vitro* incubation of spermatozoa with oviductal fluid resulted in maintenance of sperm motility (humans: ZHU et al., 1994; EVREV et al., 1994; cattle: MC NUTT et al., 1994; GRIPPO et al., 1995), presumably by providing glycolyzable substrates (ENGLE at al., 1975). A beneficial effect on sperm motility has been demonstrated using OEC conditioned medium (ANDERSON and KILLIAN, 1994; IJAZ et al., 1994; ABE et al., 1995a;b) and cocultures of spermatozoa with OEC (SUAREZ et al., 1991; GUERIN et al., 1991; BASTIAS et al., 1993; GUTIERREZ et al., 1993; THOMAS et al., 1994b; YEUNG et al., 1994; LAPOINTE et al., 1996). Recently, oviduct specific glycoproteins have been shown to support bovine sperm motility (ABE et al., 1995a; SATOH et al., 1995) and enhance oocyte penetration of hamster spermatozoa (BOATMAN and MAGNONI, 1995). Other studies revealed that binding of spermatozoa to OEC results in extended sperm longevity (LEFEBVRE and SAMPER, 1993; ELLINGTON et al., 1993b), improve fertilizing capacity (SMITH and YANAGIMACHI, 1991; POLLARD et al., 1991; CHIAN and SIRARD, 1995), increased zona binding ability (ELLINGTON et al., 1993c; ZISKIND et al., 2000) and maintained viability of spermatozoa (SMITH et al., 1987; SMITH and YANAGIMACHI, 1990). The apical plasma membrane of bovine OEC contains anchored proteinic factors that contribute to maintaining motility and viability and possibly to modulating capacitation of bovine sperm (BOILARD, et al., 2002). Exposure of sperm cells to homologous OEC or their secretory products
have resulting beneficial effect on sperm survival and motility parameters during *in vitro* culture (KERVANCIOGLU *et al.*, 1994; ZHU *et al.*, 1994; PACEY *et al.*, 1995). Co-culture of stallion spermatozoa with OEC monolayers maintained viability up to 4 days, while spermatozoa in medium alone didn’t survive more than 24 hours (SMITH and YANAGIMACHI, 1990). In addition bovine sperm membrane integrity could be maintained by co-incubating with OEC *in vitro* (POLLARD *et al.*, 1991).

### 2.2.3 Regulation of sperm capacitation

Sperm capacitation is a necessary prelude to fertilization and constitutes a set of changes in the plasma membrane that enable sperm to undergo the acrosome reaction (BLEIL and WASSERMAN, 1980, 1983). This set of changes consists of removal, addition and/or alterations of the sperm plasma membrane components. Portion of these changes occurs in the oviduct (ADAMS and CHANG, 1962; HUNTER and HALL, 1974; YANAGIMACHI, 1994). Studies in cattle (GUYADER and CHUPIN, 1991), sheep (GUTIERREZ *et al.*, 1993), hamster (SMITH and YANAGIMACHI, 1990) and horses (ELLINGTON *et al.*, 1993a,c) have shown that sperm released from OEC *in vitro* were at least partially capacitated as determined by induction of acrosome reaction or *in vitro* fertilization. Furthermore it was reported that the *in vitro* sperm-oviduct interaction in swine is initiated by uncapacitated spermatozoa binding to OEC and is continued by the induction of capacitation in cocultured spermatozoa (FAZELI *et al.*, 1999). Moreover, sperm capacitation was promoted by oviductal fluid (PARRISH *et al.*, 1989; MC NUTT and KILLIAN, 1991; MAHMOUD and PARRISH, 1996) and bovine oestrus associated protein (KING *et al.*, 1994). Oviductal fluid could induce sperm capacitation by facilitating efflux of cholesterol from the sperm plasma membrane (EHRENWALD *et al.*, 1990). However, the ratio of cholesterol to phospholipids was higher in fluid collected from the bovine oviductal isthmus compared to ampullary fluid, supporting the concept that the isthmus serves as a sperm reservoir, where cholesterol efflux would be minimized (GRIPPO *et al.*, 1994). OEC-conditioned medium (ANDERSON and KILLIAN, 1994; CHIAN *et al.*, 1995) and coculture of spermatozoa with OEC were used to promote sperm capacitation *in vitro* (sheep: GUTIERREZ *et al.*, 1993; cattle: ELLINGTON *et
Review of literatures

al., 1991; GUYADER and CHUPIN, 1991; horses: ELLINGTON et al., 1993a; humans: KERVANCI OGLU et al., 1994). Because the life span of capacitated spermatozoa is relatively short (BEDFORD, 1983; YANAGIMACHI, 1994), regulation of the rate of capacitation of spermatozoa stored in the oviductal isthmus could represent an important mechanism for ensuring availability of viable spermatozoa at the site of fertilization. It has been reported that capacitation of spermatozoa in the female reproductive tract involves changes in the sperm plasma membrane and an influx of calcium ions (SINGH et al., 1978; RUKNUDIN and SILVER, 1990). DOBRINSKI et al. (1997) concluded that maintenance of low Ca\textsuperscript{2+}, delay of capacitation and prolonged viability were observed in equine spermatozoa incubated with OEC in vitro. Changes in the sperm head membrane are also observed by SEM during in vivo interaction with the OEC and are considered as a manifestation of the completion of capacitation (HUNTER et al., 1991). It has been reported that equine spermatozoa, which are released from OEC monolayers and capacitated, can bind to the zona pellucida (ELLINGTON et al., 1993c). Moreover exposure of spermatozoa to OEC also promoted the acrosome reaction (DE JONGE et al., 1993; EVREV et al., 1994; GRIPPO et al., 1995). An explanation for this functional ambiguity of the oviducts is that sperm attachment to OEC delays capacitation while OEC secretions induce capacitation (SMITH, 1998).

2.2.4 Reduction the incidence of polyspermy

Sperm binding to OEC could limit the number of spermatozoa at the site of fertilization, while ensuring that a sufficient number is available in the oviduct when ovulation occurs. In hamster the ratio of sperm to eggs at the site of fertilization is less than 1:1 until at least half the eggs are fertilized (CUMMINS and YANAGIMACHI, 1982). When porcine sperm were surgically infused into oviducts at the pre-ovulatory stage, a high incidence of polyspermy resulted (HUNTER, 1973; HUNTER and NICHOL, 1988). HUNTER and LEGLISE (1971) found that polyspermy increased after resection of the isthmic part of the oviduct in pigs. Furthermore, the rate of polyspermy decreased when boar sperm were incubated with OEC prior to fertilization in vitro (NAGAI and MOOR, 1990; KANO et al., 1994; DUBUC and
SIRARD, 1995). Thus prevention of polyspermy may be an additional function of sperm binding to oviductal epithelium in the reservoir.

**2.3 SPERM RELEASE FROM THE OVIDUCTAL RESERVOIR**

Bovine spermatozoa can remain arrested in the isthmus for up to 18 h and detached from the epithelium near the time of ovulation and a few reaches the ampulla where fertilization takes place (HUNTER and WILMUT, 1984; SUAREZ et al., 1990; HUNTER, 1998). Moreover it was reported that release of spermatozoa from the oviductal isthmus appears to be associated with ovulation (pig: HUNTER, 1981; sheep: HUNTER et al., 1982; hamster: SMITH and YANAGIMACHI, 1989; ITO et al., 1991). On the contrary, DE MOTT and SUAREZ (1992) suggested that release of sperm from the isthmic reservoir is independent of ovulation. Evidence for a role of sperm capacitation in the release of spermatozoa from the OEC has been found in hamsters (SMITH and YANAGIMACHI, 1989; 1991), cattle (LEFEBVRE and SUAREZ, 1996) and horses (THOMAS et al., 1995a). Moreover, DOBRINSKI et al. (1997) reported that changes in the sperm surface characteristics associated with capacitation trigger the release of spermatozoa from the oviductal epithelium. A third factor implicated in the detachment of spermatozoa from OEC is the development of hyper-activated motility in oviductal spermatozoa (KATZ and YANAGIMACHI, 1980; SUAREZ, 1987; SUAREZ and OSMAN, 1987; GUERIN et al., 1991; DE-MOTT and SUAREZ, 1992; SUAREZ et al., 1992). *In vitro* studies suggested that spermatozoa may develop hyperactivated motility while bound to OEC (ELLINGTON et al., 1991; 1993b; POLLARD et al., 1991). DE MOTT et al. (1995) stated that fetuin interfered with hamster sperm attachment to the oviductal epithelium by binding to the acrosomal region of the fresh epididymal sperm. Feutin did not bind to hyper-activated sperm. Therefore, release of sperm from the oviductal epithelium may be associated with modification of sperm head surface that coincide with capacitation and/or hyper-activation.
2.4 IN VITRO SYSTEMS TO STUDY THE SPERM OVIDUCT INTERACTION

2.4.1 Tissue explants

Explants of oviductal epithelium from surgically excised oviducts or from oviducts recovered after slaughter have been used to study the interaction of spermatozoa with OEC in pigs (RAYCHOU DHURY and SUAREZ, 1991), cattle (LEFEBVRE, 1997; LEFEBVRE et al., 1995; 1997) and horses (LEFEBVRE and SAMPER, 1993; THOMAS et al., 1994a). Explants cultures were also used to characterize secretion of lipids and proteins synthesis by the OEC (HENAULT and KILLIAN, 1993; WOLDESENBET and NEWTON, 2003) and to study ciliary’s activity (GADDUM-ROSSE and BLANDAU, 1976). Within 30 min of disaggregation the clumps of epithelial cells formed everted vesicles with apical surfaces facing outward, henceforth referred to as explants (SUAREZ et al., 1991; 1998; POLLARD, 1992; THOMAS et al., 1994a; DE MOTT et al., 1995). OEC vesicles are physiologically more responsive model for the in vivo oviduct than are OEC monolayers (BUREAU et al., 2002). DE PAUW et al. (2002) have developed a new in vitro method for analysing the sperm oviductal explants binding capacity and found that bovine sperm-oviduct interaction can preferably be investigated in oviductal explants smaller than 20000 µm². The later authors added that determination of the sperm oviductal explants binding capacity could become a reliable in vitro method for predicting the NRR of a given sire.

2.4.2 Oviductal epithelial cells monolayers (OECM)

Because of their more uniform characteristics compared to tissue explants and because they can be stored frozen and are easier to handle, oviductal epithelial cell monolayers (OECM) grown in culture have been widely used to study sperm-oviduct interaction (sheep: GUTIERREZ et al., 1993; dogs: ELLINGTON et al., 1995; cattle: ELLINGTON et al., 1991; CHIAN and SIRARD, 1995; horses: THOMAS et al., 1994b, 1995a; THOMAS and BALL, 1996; humans: BONGSO et al., 1993;
KERVANCIOGLU et al., 1994; ELLINGTON et al., 1998a,b). OEC monolayers can be maintained in serum free culture (VAN LANGENDONCKT et al., 1995) and it have been characterized with regard to its cell morphology and protein secretion (cattle: JOSHI, 1991; horses: ELLINGTON et al., 1993d; THOMAS et al., 1995b,c). Cilia on OEC were generally lost after passage of OEC monolayers in culture (JOSHI, 1988, 1991; BATTUT et al., 1991; THOMAS et al., 1995c). OEC monolayers could be passaged for up to 14 -18 passages in cattle, 10 passages in rabbits, 6 passages in humans before reaching a crisis stage characterized by arrest of cell growth and alterations in cell morphology. In contrast, epithelial cell monolayers from mice couldn't be sub-cultured (OUHIBI et al., 1989). Oviductal epithelial cell monolayers have been shown to respond to the introduction of spermatozoa into the culture with a change in their secretory products (ELLINGTON et al., 1993d; THOMAS et al., 1995b). Monolayers of oviductal epithelial cells have also been used to study formation of oviductal fluid and glucose transport in rabbits (DICKENS et al., 1993; EDWARDS and LEESE, 1993).

2.5 SPERM CHROMATIN STABILITY

The item sperm chromatin means the sperm DNA and its adherent proteins. The integrity of mammalian sperm DNA is of vital importance for the paternal genetic contribution to a normal offspring and the chromatin status of the sperm is important for successful embryo development (BEDFORD et al., 1973; EVENSON et al., 1980). Furthermore, damaged DNA in the single sperm that fertilizes a female oocyte can have a dramatic negative effect on the embryo development (EVENSON, 1997; 1999a,b).

2.5.1 Sperm chromatin packaging

The nuclear structures of spermatogonia, spermatocytes and early round spermatids are similar to that observed in somatic cells. However, during mid to late spermiogenesis the spermatid nucleus undergoes transformations by two distinct processes. The first process involves reconfiguration of the nuclear matrix (BENAVENTE and KROHNE, 1985; LONGO et al., 1987; BELLVÈ et al., 1990;
HESS et al., 1993). The second process of nuclear reorganization involves replacement of the somatic cell like histones firstly with transition proteins and final addition of sperm specific protamines (CALVIN and BEDFORD, 1971; FAWCETT et al., 1971; KUMAROO et al., 1975; MEISTRICH et al., 1976; WARRANT and KIM, 1978; BALHORN, 1982; WARD and COFFEY, 1991; GREEN et al., 1994). Mammalian protamines are rich in arginine and cysteine residues, which form disulfide (S-S) bonds within and among adjacent protamine molecules. Two types of protamines, 1 and 2 have been described in sperm nuclei of eutherian mammals. However, mature bovine sperm contain only protamine-1, which typically forms two intramolecular, and three intermolecular S-S bonds (BALHORN et al., 1991). The most accepted model for how protamines interact with sperm DNA predicts that protamines lie lengthwise in the minor groove of the DNA with each positively charged arginine residue neutralizing one negative charge of the DNA’s phosphodiester backbone (BALHORN, 1982; WARD and COFFEY, 1991; PIRHONEN et al., 1994) as shown in figure 1.
Figure 1: Equivalent levels of DNA packaging in somatic cells (left) and sperm cells (right). In somatic cells, DNA is compacted into solenoids with about 6 nucleosomes per turn. In the sperm nucleus, protamines bind to the DNA, neutralizing its negative charge, and coiling the complex into tight circles these circles collapse into a "doughnut shaped structure." Each doughnut represents one DNA loop attached to the nuclear matrix. (WARD, 1993).
Recent evidence would contradict this model and suggests that protamines bind in the major rather than the minor groove of the DNA (HUD et al., 1993; PRIETO et al., 1997; BREWER et al., 1999). Regardless, binding of protamines to sperm DNA transforms the poly-anionic DNA into a stable, neutral polymer which is resistant to chemical and physical damage and is nearly 6 times more condensed than DNA found in mitotic chromosomes (POGANY et al., 1981). Mature mammalian spermatozoa contain high percentages of protamines, for example human and mouse sperm nuclei contain more than 85 % and 95 % protamines in their nucleoprotein component respectively (DEBARLE et al., 1995). In mice, protamines allow the mature sperm nuclei to adopt a volume 40 times less than that of normal somatic nuclei (WARD and COFFEY, 1991). When spermatozoa migrate through the epididymis sulphhydryl groups of the cysteine-rich protamines become oxidized resulting in large numbers of disulphide bonds between cystine residues. Such changes are thought to stabilize sperm nuclei (BEDFORD, et al., 1973; BEDFORD and CALVIN, 1974). Moreover, after ejaculation zinc enters the chromatin and binds to the free thiol groups to stabilize its quaternary structure (ARVER and ELIASSON, 1982; BJORNDAHL and KVIST, 1990). Thus stabilization of chromatin seems to compensate for the lack of DNA-repair enzymes (MATSUDA et al., 1985). Chromatin condensation is disturbed when lysine-rich somatic histones are not sufficiently substituted by arginine- and cysteine-rich protamines during spermiogenesis (MEISTRICH et al., 1978, 1976). Complete chromatin packaging is essential for normal sperm functioning (KOSOWER et al., 1992). It has been shown that incomplete replacement of histones by protamines is associated with male subfertility (AUGER et al., 1990). Stabilization is not always complete, since it has been shown that there are great differences among the spermatozoa present in any given ejaculates (KOSOWER et al., 1992). Moreover, heterogeneity of sperm nuclear maturity has been reported in different semen samples especially between fertile and infertile patients (EVENSON et al., 1980).
2.5.2 Factors affecting sperm-chromatin stability

The stability of sperm chromatin is not constant, as it can be clearly changed in an individual animal within a short period of time (BALLACHEY et al., 1987; GOGOL et al., 2002).

2.5.2.1 Age of the semen donors and aging of spermatozoa

Age of semen donors appears to be related to a significant increase in sperm DNA fragmentation (SPANO et al., 1998; EVENSON et al., 2002). Comparison of chromatin structure of sperm from two groups of bulls aged 14 months and 4 years indicates that this parameter improves with the bull's age (KARABINUS et al., 1990). In addition, studies involving a large group of men showed the age of semen donors to be strongly correlated to sperm chromatin structure (SPANO et al., 1998). Moreover, decreased sperm chromatin stability was found in ejaculates taken from male rabbits less than 5 months and more than 20 months of age (GOGOL et al., 2002).

A further factor which affects the sperm chromatin stability is the long sexual abstinence (EVENSON et al., 1991; SPANO et al., 1998). The semen samples of rabbits, bulls and sheep, which were collected outside of the breeding season, had showed increased chromatin instability and less fertility than those collected during the breeding season. This might be due to over-maturation of spermatozoa during long storage in the epididymis (MILLER and BLACKSHAW, 1968; SALISBURY and HART, 1970; RODRIGUEZ et al., 1985). Furthermore, sperm aging in vitro also results in increased susceptibility of sperm DNA to denaturation. ESTOP et al. (1993) demonstrated that mouse sperm aged in vitro showed chromatin denaturation within one hour of incubation at room temperature.

2.5.2.2 Temperature

A further essential cause for the increased occurrence of unstable sperm chromatin is an increase of both internal body temperature and ambient temperature (THIBAULT et al., 1966; STONE 1977). The patients suffering from cryptorchidism (the testicles lie in the abdominal cavity) are infertile, because the higher abdominal
temperature disturbs the spermatogenesis process (CREW, 1922). Moreover, the portion of spermatozoa with unstable chromatin was clearly increased in individuals with feverish illnesses and showed likewise fertility disturbances (GUNN et al., 1942; EVENSON et al., 2000).

2.5.2.3 Cryoconservation

The influence of cryoconservation procedure on the sperm chromatin status is controversially discussed. EVENSON et al., (1994) estimated that neither the process of the cryoconservation nor the shock freezing of ejaculates had an influence on the ultrastructure or stability of sperm chromatin, whereas others observed a degradation of the chromatin stability particularly with subfertile individuals (HAMMADEH et al., 1999; 2001; BLOTTNER et al., 2001). KARABINUS et al. (1990) stated that incubation of bull sperm in cryoprotectant media increased the susceptibility of DNA to denaturation in situ within 30 minutes. ROYERE et al. (1988) and HAMAMAH et al. (1990) claimed that a relationship existed between an "over-condensation" state for frozen-thawed sperm chromatin and a lower conception rate for human semen after cryostorage. ROYERE et al. (1991a) suggested that freeze-thawing procedures might alter the DNA / nuclear protein relationships and impair the fertilizing ability of human sperm. In addition, frozen-thawed boar spermatozoa showed significantly increased (P < 0.05) chromatin compactness compared to fresh spermatozoa. Moreover CORDOVA et al. (2002) found that the percentage of spermatozoa with unstable chromatin was significantly (P < 0.05) higher in frozen semen samples than that found in fresh semen.

2.5.2.4 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are harmful to sperm at elevated levels (JONES and MANN, 1973; ALVAREZ et al., 1987; AITKEN et al., 1989a,b; 1992; D'AGATA et al., 1990; AITKEN and Fisher, 1994; CUMMINS et al., 1994; BECKMAN and AMES, 1997; ARMSTRONG et al., 1999; EVENSON et al., 2002) and are a major cause of damage to sperm DNA (GAGNON et al., 1991). The major sources of ROS in diluted semen incubated at ambient temperature are oxidative de-amination of aromatic
amino acids by aromatic L-amino acid oxidase released from dead and damaged sperm (SHANNON and CURSON 1972; 1981), mitochondrial respiration (AITKEN and CLARKSON, 1987), and seminal leukocytes (AITKEN et al., 1992; KESSOPOULOU et al., 1992; ALVAREZ et al., 2002). Because sperm are almost devoid of cytoplasm, they possess only very low amounts of the ROS-scavenging enzymes that protect somatic cells from oxidative damage. Moreover DNA repair enzymes are apparently inactive in mature sperm making these cells more susceptible to oxidative attack (HUGHES et al., 1998). Functional sperm rely on the tight packing of their DNA around protamines, which reduces exposure to free radicals and on antioxidants present in the seminal plasma for protection from oxidative damage (HUGHES et al., 1998). During in vitro manipulation of sperm samples oxidative damage to sperm DNA can be alleviated by supplementing the diluent with antioxidants (HUGHES et al., 1998), ROS-degrading enzymes and elimination of oxygen from the diluent (SHANNON and CURSON, 1982).

2.5.2.5 Trace elements and other factors

Zinc and copper are trace elements, which play an important role in the stability of sperm cells chromatin by stabilization of the free thiol group. The Prostate gland secretion is rich with zinc, so that the sperm chromatin is protected when mixed with seminal plasma during ejaculation. A lack of zinc leads to increased susceptibility of the sperm chromatins to in situ denaturation (BLAZAK and OVERSTREET, 1982; RODRIGUEZ et al., 1985). Some therapeutically used chemicals (SHALET, 1980; EVENSON et al., 1999), environmental pollution stress (WYROBEK et al., 1997; LEMASTERS et al., 1999; PERREAUET et al., 2000; SELEVAN et al., 2000), cigarette smoking (SPANO et al., 1998) and cancer diseases (EVENSON and MELAMED, 1983; EVENSON et al., 1984; FOSSA et al., 1997) are also factors, which negatively affect the stability of sperm chromatin. It is noteworthy that a partial decondensation state of human sperm chromatin may occur during in vitro capacitation. However, beyond some level of decondensation the fertilizing ability could be altered (ROYERE et al., 1991b).
2.5.3 Evaluation of sperm chromatin stability

In assessing semen quality, animal and human fertility clinics typically measure sperm density, motility and morphology. Clinics rarely measure sperm DNA integrity, primarily because they are unaware of the availability a rapid, reliable and practical test. The methods of studying sperm chromatin status includes: aniline blue (AB), which indicates the presence of excessive histones (TERQUEM and DADOUNE, 1983), Chromomycin A3 (CMA3), which shows protamine deficiency (IRANPOUR et al., 2000), comet assay, which shows extent of DNA fragmentation (HUGHES et al., 1999) and acridine orange (AO), which reflects chromatin resistance to denaturation (TEJADA et al., 1984).

2.5.3.1 Sperm chromatin structure assay (SCSA)

Acridin orange (AO) intercalates into double-stranded (ds) DNA as a green fluorescing monomer and binds to single-stranded (ss) DNA as a red fluorescing aggregate when excited by a blue laser light (488nm) (ICHIMURA et al., 1971). The SCSA was developed to measure sperm DNA susceptibility to in situ acid induced denaturation by quantifying the metachromatic shift from green fluorescence of AO bound to ds-DNA to red fluorescence emitted by AO bound to ss-DNA (EVENSON et al., 1980). Two DNA denaturation methods using AO were used, one combined from the earlier recommendations of ROSCHLAKU, (1965) and RIGLER, (1966) (RRAO method), which used earlier for in situ detection of apoptotic cells, and the other method suggested by TEJADA et al. 1984 (TAO method) which used for sperm cells. The SCSA is an adaptation of the two-steps AO procedure originally designed by DARZYNKIEWICZ and colleagues (1975) for simultaneous measurements of DNA and RNA content in somatic cells. Whatever minute amounts of RNA may be present in a mature sperm do not interfere with SCSA data. It is of interest, but not understood that this procedure denatures protamine associated DNA in sperm but does not denature somatic cell DNA associated with histones (EVENSON et al., 1985).
2.5.3.2 Modified fluorescence microscopical SCSA (mf-SCSA)

TEJADA and co-workers (1984) developed the first modification of the SCSA for the conventional fluorescence microscope to eliminate the necessity of flowcytometry for the assessment of human sperm chromatin stability with AO fluorescence. This simplified method termed acridin orange TEJADA (AOT) and based upon the same principles as SCSA, but relies on human visual interpretation of the fluorescent characteristics of AO intercalated into the sperm nucleus. The major benefit of microscopical approach to SCSA is a simultaneous evaluation of sperm chromatin status and morphology. ANGELOPOULOS et al. (1998) observed a high consistency between the mean percent of morphologically normal spermatozoa and the percent of green sperm determined through AOT. Moreover, DOBRINSKI et al. (1994) found a significant correlation between red fluorescing cells and the percentage of pyriform heads and vacuoles. However, they also found that high numbers of abnormally condensed nuclei could be detected in the absence of other defects. Several laboratories have used the AOT technique to assess male fertility potential (IBRAHIM and PEDERSEN, 1988; ROUX and DADOUNE, 1989; CLAASSENS et al., 1992). Significant correlations were recorded between red fluorescence and abnormal sperm morphology, but not between red fluorescence and motility (TEJADA et al., 1984; IBRAHIM and PEDERSEN, 1988). This suggests a potential infertility factor in spermatozoa that is removed from sperm viability alone. There have been problems reported with the interpretation of AO fluorescence on sperm cells using AOT. Rapidly fading fluorescence and indistinct colour are most commonly associated with this technique (DURAN et al., 1998; CLAASSENS et al., 1992). Others have modified the technique to enhance the stability of the stain across a variety of species (DOBRINSKI et al., 1994; BELETTI and MELLO, 1996). KOSOWER et al. (1992) noticed that the acridine orange fluorescence of sperm nuclei is determined by the thiol/disulfide status of DNA-associated protamines. Their results indicate that sperm nuclei treated with acetic alcohol show a green fluorescence when their nuclear protamines are rich in disulfide bonds and show red fluorescence when their protamines are poor in disulfide bonds (KOSOWER et al., 1992). This suggests that the structure of chromatin is important for controlling the interaction of AO with DNA.
This finding strengthens the SCSA argument that varying degrees of susceptibility to acid denaturing conditions are indicative of abnormal chromatin structure (EVENSON et al., 1985). Perhaps the use of thiol-protectant chemicals such as dithiothreitol or 2-mercaptoethanol may help stabilize the chromatin after acid treatment and minimize the present constraints associated with the AOT technique, i.e. rapid quenching and/or shifts in fluorescence color over time.

### 2.5.4 The sperm chromatin status and fertility

The SCSA has been used to establish a relationship between fertility and sperm chromatin stability in cattle (EVENSON et al., 1980; BALLACHEY et al., 1988; DOBRINSKI et al., 1994), humans (EVENSON et al., 1980; EVENSON, 1999a), swine, (EVENSON and JOST, 1994), mice, (BALLACHEY et al., 1986) and horses (KENNEY et al., 1995). Several studies have shown strong negative correlations between the SCSA variables and bull fertility as measured either by non-return rate of the female (KARABINUS et al., 1990) or the competitive index based on heterospermic performance among bulls (BALLACHEY et al., 1988).

Chromatin defected spermatozoa are able to fertilize the oocyte, however results in high rate of early embryonic mortality (TEJADA et al., 1984; GORCZYCA et al., 1993; DARZYNKIEWICZ et al., 1997; EVENSON et al., 2000). EVENSON et al. (1980) found that sperm of bulls, mice and humans with low or questionable fertilization ability shows a significant reduction of the DNA resistance to in situ denaturation compared with semen from normal fertile individuals. KENNEY et al. (1995) stated that morphologically abnormal spermatozoa of subfertile horses possess nearly unstable chromatin. Furthermore it was estimated that many of the morphologically normal sperm of subfertile individuals exhibited increased susceptibility of their DNA to denaturation in situ indicating that the entire sperm population of these ejaculate is qualitatively inferior (TEJADA et al., 1984; BALLACHEY et al., 1988; LOPES et al., 1998). It was reported that reduced sperm chromatin stability as measured by SCSA correlates strongly with DNA strand breaks (ARAVINDAN et al., 1997) and subfertility in bull, human and boar (BALLACHEY et al., 1987; 1988; EVENSON et al., 1994; 1999). Moreover it was estimated that DNA fragmentation of human
spermatozoa is negatively correlated with in vitro fertilization outcome (SUN et al., 1997; LOPES et al., 1998; EVenson et al., 1999). It was concluded that sperm morphology and protamine deficiency independently affect fertilization rate (ESTERHUIZEN et al., 2000a,b; NASR-ESFAHANI et al., 2001). On the contrary, SPANO et al. (1999) observed no relationship between chromatin stability and fertilization rate, suggesting that this difference was due to methodology of acridin orange assessment. ANGELOPOULOS et al. (1998) stated that AO staining did not predict fertilization efficiency or pregnancy outcome in IVF cycles.

2.5.5 Efficiency of SCSA to predict fertility

SCSA data on thousands of semen samples from bulls (BALLACHEY et al., 1987; 1988; EVenson, 1999a,b), stallions (LOVE and KENNEY, 1998; EVenson and JOST, 2000), human (SUN et al., 1997; LOPES et al., 1998) and boars (EVenson et al., 1994) showed the clinical value of this assay for animal fertility assessment. Some investigators suggested that sperm from subfertile men showed an increase in red fluorescence (EVenson et al., 1980; TEJADA et al., 1984; LIU and BAKER, 1992). Moreover, it was concluded that spermatozoa that fertilize the oocytes in vivo and in IVF were limited to whose nuclei exhibited green AO fluorescence (HOSHI et al., 1996). The SCSA was found to be a useful indicator of fertility because of its ability to evaluate a parameter of the spermatozoa that cannot be assessed by traditional tests of sperm quality such as motility and morphology (LOVE and KENNEY, 1998). In addition, the SCSA may be a valuable complement for routinely practiced microscopic evaluation of sperm morphology of AI bull semen (JANUSKAUSKAS et al., 2000), since the morphologically abnormal spermatozoa might possess DNA strand breaks (SAKKAS et al., 1999), as well as abnormal chromatin structure (SAILER et al., 1996). In other studies a weakly correlation between SCSA and conventional spermatological parameters were recorded (GORCZYCA et al., 1993; DARZYNKIEWICZ et al., 1997; BOCHENEK et al., 2001; EVenson et al. 1991, 1999). Semen samples with normal conventional parameters may have very poor DNA quality that contributes to infertility. Therefore, the SCSA test offers additional clinical information not provided by conventional semen analysis.
alone. On the contrast, AO staining did not predict fertilization efficiency or pregnancy outcome in IVF cycles (ANGELOPOULOS et al., 1998). Furthermore, the ability of the SCSA to predict fertilization and pregnancy outcome after in vitro fertilization (IVF) is controversially discussed (CLAASSENS et al., 1992; LIU and BAKER, 1992; EGGERT-KRUSE et al., 1996a; HOSHI et al., 1996).

2.6 SPERM MEMBRANE INTEGRITY

The sperm plasma membrane is the outer cell structure that acts as a physiological barrier and its functional and structural integrity are required for their normal activities.

2.6.1 Structural integrity of sperm membrane

Several methods have been used to distinguish between viable and non-viable cells. The sperm vital stains such as eosin/nigrosin (EN) and trypan-blue (TB) have been used for decades to evaluate plasmalemma (sperm outer membrane) integrity (HANCOCK, 1951; MAYER et al., 1951; SWANSON and BEARDEN, 1951; HACKETT and MACPHERSON, 1965). Staining with EN has been combined with Giemsa and this method is reliable and simple enough for routine work (TAMULI and WATSON, 1994). In addition, nucleic acid stains such as Hoechst 33258 have been combined with fluoresceinated lectins (MORTIMER et al., 1990; CASEY et al., 1993; VALCARCEL et al., 1997), or with other fluorescent probes such as chlortetracycline to assess capacitation and acrosomal exocytosis (FRASER et al., 1995; WANG et al., 1995). Combination of fluorescent probes can be used to assess sperm viability (GARNER et al., 1986). Alternatively by combining a probe that distinguishes between viable and non-viable cells with another probe that stains the acrosomal contents or outer acrosomal membrane, both sperm viability as well as acrosomal status can be assessed (TAO et al., 1993; MAXWELL and JOHNSON, 1997; COOPER and YEUNG, 1998; HARKEMA et al., 1998).

The fluorescent stain Propidium Iodide (PI) is a DNA-specific red fluorescent stain that does not penetrate intact plasmalemma and can be combined with fluorescent stains that penetrate also intact plasma membrane, like carboxyfluorescein diacetate
Review of literatures

(CFDA; GARNER et al., 1986; HARRISON and VICKERS, 1990) and SYBR-14 (GARNER and JOHNSON, 1995). GARNER et al., (1994) developed a double, supravital stain consisting out of PI and SYBR14. The two stains, SYBR-14 (a green, membrane-permeable stain) and PI (a red, membrane-impermeable counter stain) have the same cellular target (sperm DNA). A direct comparison among vital stains and the HOST for their ability to predict fertility has not been reported, albeit results of the HOST and SYBR/PI staining have been shown to be well correlated with non return rates (NRR) after artificial insemination (AI) in two independent studies (CORREA et al., 1997; JANUSKAUSKAS et al., 2000).

2.6.2 Functional integrity of sperm membrane

The ability of the sperm tail to swell and/or coil in the presence of the hypoosmotic solution demonstrates that the influx of water across the membrane occurs normally (JEYENDRAN et al., 1984; KIEFER et al., 1996; BILJAN et al., 1996). DREVIUS and ERIKSSON (1966) and DREVIUS (1972) demonstrated that sperm cells do swell in hypo-osmotic environment and that over a relatively wide range of tonicities they behave as perfect osmometers (swelling is proportional to the degree of hypotonicity). This phenomenon indicates the normal plasma membrane integrity of spermatozoa. Furthermore, the percentage of swollen cells within a sperm subpopulation has been suggested to be an indicative parameter for the membrane biochemical activity and fertility of human spermatozoa (JEYENDRAN et al., 1984).

2.6.2.1 The hypo-osmotic swelling test (HOST)

In the classic hypo-osmotic swelling test, the cells are classified subjectively by morphologic evaluation as swollen or non-swollen using phase contrast microscopy after fixation and cells with swollen tails are supposed to be membrane-intact (JEYENDRAN et al., 1984). The HOST provides information regarding the membrane integrity of the sperm tail, but it has been shown that the membranes at the head and the tail compartments have different liability (HAMMERSTEDT et al., 1979). Since spermatozoa appear to behave as perfect osmometers (DU et al., 1994; GILMORE et al., 1996), changes in cell osmole content, as well as the distribution of the
osmotically active and inactive cells within a sperm population might be revealed as volume changes of sperm cells under iso-osmotic conditions. However, information about changes in cell osmole content might be more detectable under hypo-osmotic stress, since this would induce a rapid increase of cell volume due to the high water permeability of the spermatozoa (GILMORE et al., 1996).

2.6.2.2 Modified hypo-osmotic swelling test (m-HOST)

An alternative version of HOST is the m-HOST in which not only the number of swollen sperm but also both the extent of swelling and volume distributions of the sperm cell populations can be objectively measured by means of an electronic cell counter called CASY1 (PETZOLDT, 1988; ENGEL and PETZOLDT, 1994; PETZOLDT and ENGEL, 1994). CASY1-measurements are based on the pulse frequency analysis providing high precision and resolution of data. It is possible to differentiate between sperm cell subpopulations in the cell volume distribution curves. Among volumetric parameters, which can be derived from CASY1, the relative volume shift (RVS) of the modal value of the volume distribution curve and regulative volume decrease (RVD). RVS was suggested to be an indicative parameter of the membrane integrity of human sperm (ENGEL and PETZOLDT 1994; PETZOLDT and ENGEL 1994). Intact cells swell in response to hypo-osmotic conditions, following that a cell volume reduction occurs (STRANGE et al., 1996). RVD under hypo-osmotic conditions as described for somatic cells has been documented in bovine sperm (KULKARNI et al., 1997). The RVD is an important physiological function as shown by studies of sperm from c-ros tyrosine kinase receptor knockout mice (YEUNG et al., 1999).

2.6.3 Efficiency of the HOST to predict fertility

Different clinical studies indicate that the HOST as a single assay is not sufficient to predict the fertilizing capacity of an ejaculate (JEYENDRAN et al., 1992; VAN DEN SAFFELE et al., 1992; SALLAM et al., 2003). Furthermore, no significant correlation was observed between sperm swelling and in vitro sperm fertilizing capacity as assessed by the zona-free hamster oocyte penetration assay (SMITH et al., 1992).
Studies have been performed to evaluate the correlation of subnormal HOST scores with other semen parameters that are believed to be predictive of fertilization potential, albeit with various conclusions (CHAN et al., 1985; 1991; WANG et al., 1988; COETZEE et al., 1989; MORDEL et al., 1989; FUSE et al., 1991; OSTERHUIS et al., 1996; BUCKETT, 2003). Combining HOST results with other spermatological parameters (e.g. motility and vitality) was useful in order to improve correlations with the fertility rate in vitro (VAN DER VEN et al., 1986; MCCLURE and TOM, 1991; RAMIREZ et al., 1992) and conception rate in human (ASCHKENAZI et al., 1992). Although a couple of studies have found lower fertilization rates with subnormal HOST scores (VAN DER VEN et al., 1986; AVERY et al., 1990; HAUSER et al., 1992; ABU-MUSA et al., 1993; TARTAGNI et al., 2002), the majority have found HOST to be one of the least useful tests to determine low fertilization potential of sperm (BARRATT et al., 1989; SJOBLUM and COCCIA, 1989; AVERY et al., 1990; CHAN et al., 1990; ENGINSU et al., 1992; KIEFER et al., 1996). Recently a defect in sperm volume regulation has been identified as the cause of sterility in the protein tyrosine kinase receptor c-ros knockout mouse (YEUNG et al., 1999, 2000). Positive correlations between the results of HOST and hamster egg penetration assay were observed for specimens with high or low fertility rates (i.e. ejaculate with low swelling rate demonstrated low fertility), but not always in the middle range of rates (OKADA et al., 1990). Moreover, the HOST can assist in evaluating semen quality, judged by the fertilization rate in an in vitro fertilization program (YAVETZ et al., 1995). It was reported that the HOST was not correlated with percentage of pregnant mares but showed a tendency to correlate with the number of services per pregnancy; therefore it could be an additional method for evaluating stallion fertility (NEILD et al., 1999; 2000). MCCLURE and TOM (1991) found no significant correlations between HOST and fertility for spermatozoa from infertile men. Moreover, in cattle, a preliminary report using five bulls found that the hypo-osmotic swelling test was not related to IVF success (ROTA et al., 2000). It is noteworthy that the HOST can accurately evaluate viability in fresh human spermatozoa but not in cryopreserved ones (ESTEVES et al., 1996).
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The used chemicals and reagents from which the buffers, solutions, and media were made as well as all labour objects are presented in an appendix.

This study was applied at Institute for Reproductive Medicine, School of Veterinary Medicine HANNOVER, Bünteweg 15, D-30559 HANNOVER, GERMANY and Institute of Animal Science and Animal Husbandry, Federal Agricultural Research Centre (FAL), 31535 NEUSTADT-MARIENSEE, GERMANY in the period from September 2000 to April 2004.

3.1 SEMEN SOURCE

The Rinderproduktion Niedersachsen (NORDRIND), GmbH BREMEN - HANNOVER, Lindhooper Str.110, 27283, VERDEN, generously provided the straws of cryopreserved semen from 30 bulls. The spermatozoa were processed and packed in 0.25 ml capacity mini-straws (approximately 20-30 x 10^6 sperm/straw) and stored at -196 °C in liquid nitrogen.

3.2 HANDLING OF FROZEN-THAWED SEMEN

Unless otherwise stated elsewhere, individual straws were thawed rapidly in a warm water bath at 38 °C for 30 sec. Straws were dried thoroughly to avoid the possibility of water contacting the semen when the straw is opened. Moreover fluctuations in the temperature of thawed semen were avoided to minimize the risk of chilling injury.

3.3 TRADITIONAL SPERMATOLOGICAL PARAMETERS

3.3.1 Motility parameters

3.3.1.1 Subjective sperm motility

Individual straws from 90 ejaculates (three ejaculates per bull) were thawed and the contents of each straw were separately transferred into a pre-warmed 1.5-ml
eppendorf plastic tube. About 5-µl aliquot of thawed semen was placed on a pre-
warmed glass slide then covered with a pre-warmed coverslip (18x18 mm).
The prepared slides were viewed under 160x magnifications using a phase-contrast
microscope equipped with a 38 °C warmed stage (Zeiss, JENA / GERMANY).
Progressive forward- and local- as well as non-motile spermatozoa were estimated to
the nearest 5 %. The rest of the semen samples were used for sperm morphology
assessment.

3.3.1.2 Assessment of motility using cell motion analyzer (CMA)

Computer-assisted sperm motility analysis was performed using Stromberg Mika Cell
Motion analyser (SM-CMA, Strömberg-Mika; Bad FEILNBACH, GERMANY) as
described previously by RODRIGUEZ-MARTINEZ and BERROSTEQUIETA (1994).
For motility analysis, about 8 µL aliquots of thawed semen were placed on a pre-
warmed (38 °C) Mika cell counting chamber (10 µm depth) and covered with a
special cover-slip, then the spermatozoa were visualized under a phase contrast
objective (Ph 2) at 200x magnification. For each attempt, several microscopic fields
(sequences) were analysed including at least 200 spermatozoa. The proportion of
total motile (TM) and linearly motile (LM) spermatozoa, straight-line velocity (VSL,
µm/s), curvilinear velocity (VCL, µm/s), and the average-path velocity (VAP, µm/s)
were determined. The computer settings were adjusted according to the
manufacturer’s instructions as follows: number of frames per analysis: 32, time
between two video half pictures for detection of immotile objects: 20 ms, cell size
range: 35-300 pixels, threshold value for velocity to be classified as immobile objects:
10 µm/sec, threshold value for velocity to be accepted as locally motile spermatozoa:
25 µm/sec, maximum value for linearity, 90 %, minimum number of frames, 15,
velocity class width, 5 µm/sec, maximum radius for circles: 25 µm.

3.3.2 Morphological abnormalities of spermatozoa

In this experiment, 90 ejaculates from 30 bulls (3 ejaculates per bull) were examined.
The frequencies of proximal and distal protoplasmic droplets, loose heads, acrosomal
abnormalities, abnormal mid-pieces and coiled tails were estimated after counting 200 spermatozoa in wet smears. About 160 µl aliquots of thawed semen were transferred into Eppendorf tubes containing 250 µl formol-citrate 4 %. After good mixing, wet smears were made by placing about 2 µl of semen mixture on a glass slide then gently covered with a cover slip. The prepared wet semen smears were examined using phase contrast (Ph 2) microscopy at 1000x magnification under oil immersion lens. Sperm cells abnormalities were classified according to KRAUSE (1965) as shown in figure 2.
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Acrosome abnormalities
1) Swelled acrosome
2) Acrosome in detaching
3) Detached acrosome
4) Deformed acrosome
5) Persistent acrosome granulome

Head abnormalities
6) Deformed
7) Small
8) Narrow
9) Dwarf
10) Large

Doubled Abnormalities
11) Broken neck (detached head)
12) Paraxial tail attachment

13) Retro-axial tail attachment
14) Proximal protoplasmic droplet

Mid-piece abnormalities
15) Protoplasmic droplet

Main-&end-tail piece abnormalities
16) Looped tail
17) Rolled tail
18) Tail rolled around the head

Doubled Abnormalities
19) Distal protoplasmic droplet
20) Rudimentary Tail
21) Twin head and one tail
22) One head and Twin tail

Figure 2: Forms of morphologically abnormal bull spermatozoa (KRAUSE 1965).
3.4 ADVANCED SPERMATOLOGICAL PARAMETERS

3.4.1 Viability assessment using LIVE/DEAD® Sperm Viability Kit

Both morphological integrity of sperm membrane (sperm viability) and mitochondrial activity of thawed spermatozoa were evaluated using LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Mol. Biol. Tech., GÖTTINGEN). It is composed of SYBR-14, which is a newly developed fluorescent nucleic acid stain, maximally absorbs at 488 nm and emits at 518 nm when bound to DNA of alive spermatozoa. It stains the nuclei of living sperm bright green and Propidium Iodide stain (PI) which stains the nucleic acid molecules of dead and membrane damaged spermatozoa red by intercalating into them. PI excites at 536 nm and fluoresces at 617 nm. The method described by GARNER and JOHNSON (1995) with some modifications was used as a model in this experiment.

Hepes buffer saline (HBS, see appendix) was prepared, then divided into 10 ml portions in closed plastic test tubes and kept deep frozen (-23 °C) till used. At the day of the experiment, one portion of the deep frozen HBS was thawed at room temperature. To each 10 ml portion, 0.0238 g Hepes and 0.01 g Bovine Serum Albumin (BSA) were being added, after that the pH was adjusted to 7.4 with 0.1 N NaOH. 500 µl aliquots of the final HBS solution were transferred to pre warmed Eppendorf tubes. The SYBR-14 / PI working solution was thawed at room temp. Individual frozen semen straws from each ejaculate were thawed in warm water bath (38 °C / 10 sec) and the contents were transferred to the pre-warmed 500-µl HBS aliquots. From the HBS-semen mixture, 500 µl aliquots were taken into another pre-warmed Eppendorf and mixed with 0.17 µl SYBR-14 and 2 µl PI (10 µM SYBR-14 and 120 µM PI). The samples were then gently mixed and incubated at 38 °C for 15 min. The semen smears were made in dark room by placing 20 µl aliquot of stained semen on a warm slide and smeared with the aid of another warm slide. The smears were quickly air dried and evaluated as soon as possible using a phase contrast florescence microscope (Phase 2), at 200x magnifications and 490 nm excitation filter (Leitz Laborlux-11; JENA, GERMANY). With the aid of digital camera (Olympus,
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HAMBURG) and Software analysis® 3.0 (Soft Imaging System GmbH, MÜNSTER), several fields were photographed and saved for later evaluation. A minimum of 200 spermatozoa were counted and classified as alive and membrane intact when stained green or as dead and membrane damaged when stained green–red or just red.

3.4.2 Assessment of the functional sperm membrane integrity using modified hypo-osmotic swelling test (m-HOST)

Biochemically active sperm is required for the process of capacitation, acrosome reaction and binding of the sperm cell to both oviduct epithelium and oocyte surface. The frequency of frozen thawed bull spermatozoa with functionally intact plasma membranes can be determined using simple and practical osmotic resistance tests based up on their behaviours when exposed to hypo-osmotic solutions (JEYENDRAN et al., 1984). An alternative version of this test is the modified hypo-osmotic swelling test (PETZOLDT, 1988; PETRUNKINA et al., 2001c,d).

Electronic cell counter and analyser system “CASY1” (Coulter technique employing the “pulse area analysis” signal-processing technique; Schärfe System REUTLINGEN, GERMANY) was used for assessment of the functional integrity of sperm membrane (figure 3).
3.4.2.1 General physical principles of measurement

The suspended spermatozoa are introduced into the measuring unit through a capillary of predefined geometry at a constant-stream velocity. During the measurement, an electric current is supplied to the capillary via two platinum electrodes, so the capillary filled electrolyte has a defined electrical resistance. While passing through the capillary, the cells displace electrolyte solution in proportion to cell volume. Because of the intact cells have isolation properties, resistance along the capillary rises and producing signal. This signal is scanned by CASY1 with a frequency of one million measurements per second in a low voltage field. CASY1 captures the amplitude and width of the pulse and determines the integral of the measuring signal (pulse area analysis). The resulting signal of every individual cell is analyzed in area, height, width and course of time. The cell signals are cumulated and assigned in a calibrated multi-channel analyzer that has 512,000 channels.
According to Ohm's law:
The electric voltage \( U \) = electric resistance\( (R) \) x electric current \( (I) \).
Because of the stability of the electric current \( (I) \), any changes in the electric
resistance will be accompanied by changes in the electric voltage. \( U = f \times R \). While
the electrolyte has a defined electrical resistance and the cells displace electrolyte
solution in proportion to cell volume, so that the volume of the cells was derived from
the electric resistance \( (R) = f \times V \).
From it, this equation resulted: \( U = f \times V \).

3.4.2.2 Calibration of CASY1 by latex beads

Because the two test solutions (iso-osmotic and hypo-osmotic) had different electrical
conductivities due to different concentrations of electrolyte, it was necessary to apply
a correction factor for the data recorded under hypo-osmotic conditions. This factor
was obtained by comparison of volume measurements of standard latex beads (3.4
µm in diameter, Sigma AG) in three HBS solutions of different osmolalities (150-, 300-, 450 mOsmol/Kg\(^{-1}\)) at 38 °C. The volumetric parameters of latex beads
measured at different osmolalities were fitted to the linear regression model and the
obtained factor was used to calculate the real cell volume under hypo-osmotic
conditions. The calculated correction factors were 1.16 for modal values under hypo-
osmotic conditions (180 mOsmol/kg\(^{-1}\)) and 1.11 for mean sperm cell volume.

3.4.2.3 Preparation of semen samples

Individual frozen semen straws from each ejaculate were thawed in water bath. The
thawed semen was passed through discontinuous Percoll gradients.

Preparation of discontinuous Percoll\(^\circledR\) gradients:
Percoll\(^\circledR\) (Pharmacia, UPPSALA, SWEDEN) gradients were prepared according to
the protocol described by HARRISON et al., (1993), (see appendix). The washing
method was carried out according to the protocol of PARRISH et al., (1995) with
some modification. Briefly, in obliquely positioned (45° angle), 15 ml capacity, conical
bottom glass test tube, 2 ml 90 % Percoll was placed, and over these, another 2 ml
45 % Percoll was smoothly flowed on the inner side of the tube, then allowed
warming up. The content of each straw was carefully placed on the top of the prepared discontinuous Percoll layers, and then centrifuged for 15 min at 700 g (1200 rpm). After that Percoll layers were carefully withdrawn and the sperm pellets were resuspended, each in 0.3 - 0.5 ml warm (39°C) sperm-TALP (Tyrode Albumin Lactate Pyruvate medium; PARRISH et al., 1988) presented in Eppendorf tubes, and then embedded in cavities found in a thick sponge at room temp (figure 4).

Figure 4: Washing of frozen-thawed bull semen through a discontinuous percoll gradient, a) before centrifugation; b) after Centrifugation.

From the preparatory work and other publications (PETRUNKINA et al., 2001c,d; PETRUNKINA and TÖPFER-PETERSEN, 2000) it has been found that the best sperm volume distribution curves were obtained when the sperm cell concentration per 200 µl measuring sample volume was between 20,000 and 40,000 sperm cell. Therefore, the sperm cell concentration was determined using Thoma cell counting chamber to calculate how many µl semen suspensions will be needed for 6 ml HBS to obtain a final concentration of 100,000-200,000 sperm / ml (i.e. 20,000-40,000 sperm /200 µl sample measuring volume).
3.4.2.4 Volumetric measurement

Hepes buffered saline (HBS) without polyvinyl alcohol and polyvinyl pyrrolidon was used as sperm incubation medium in the current experiment (see appendix). Two osmolalities were used, iso-osmotic (300 mOsm kg\(^{-1}\)) and hypo-osmotic (180 mOsm kg\(^{-1}\)). The pH of both solutions was adjusted to 7.4 using 1N NaOH solution, and then passed through a 0.22-µm sterile filter (Sartorius, AG, GÖTTINGEN, GERMANY) before use.

At the day of experiment, 10 ml capacity snap-cap glass vials (Schärfglas: Landgraf Laborgeräte, Langenhagen) were labeled with the name of bull, osmolarity, duration of incubation and filled with 6 ml HBS solution, then incubated at 38 °C. 2 - 4 µl samples were taken from the percoll washed semen suspension and added to 6 ml iso-osmotic (300 mOsm kg\(^{-1}\)) and hypo-osmotic (180 mOsm kg\(^{-1}\)) HBS solution contained in snap-cap glass vials pre-incubated at 38 °C. After an exactly determined incubation periods (5 and 20 min), the samples were passed through a CASY1 cell counter. At each sampling time-point, such distributions were collected from a single iso-osmotic dilution and a single hypo-osmotic dilution using a CASY1 sample volume setting of 200 µl and a size scale of 10 µm. The cursors were fixed at positions 2.3 µm and 6 µm during the entire experiment to collect all representative volume distribution fractions under different osmotic conditions.

In each sampling the data were obtained from more than 20,000 sperm cells. The volume distributions were measured at 5 min and 20 min of incubation in both iso- and hypo-osmotic HBS solutions. The incubation time in the measuring solutions remained constant for all samples.

3.4.2.5 Analysis of derived volumetric parameters

The original cell-counter data were recorded for 512,000 volume channels. To analyse and save the files, the data were formatted for 1024 effective diameter channels. Modal value of the volume distribution curve was taken into consideration, as the modal value is the most frequent value of a distribution, and are very stable against the extreme-low and high values in the distribution. Moreover it was found to be a more sensitive parameter of volume change than the mean value.
(PETRUNKINA and TÖPFER-PETERSEN, 2000). The modal values of the sperm volume distribution curves (figure 5a & 5b) were submitted to statistical analysis after correction of such values obtained under hypo-osmotic conditions with the calculated correction factor. The relative volume shift (RVS) was used as a measure of the sperm volume regulation in response to hypo-osmotic stress (PETZOLDT and ENGEL, 1994).

It was defined as: $RVS = \frac{V_{h5}}{V_{i5}}$. Where $V_{h5}$ is the modal value of the hypo-osmotic volume distribution of samples incubated for 5 min (figure 5), and $V_{i5}$ is the modal value of the iso-osmotic volume distribution after 5 minutes of incubation. When several sperm subpopulations contributed to a distribution, the values pertaining to the largest osmotically active subpopulation were used. A cell subpopulation was considered osmotically active if its RVS was $> 1$ (PETRUNKINA et al., 2000). Regulative volume decrease (RVD) of modal values of volume distribution curves was also used as an evaluation parameter of the functional integrity of sperm membrane. It was defined as; $RVD = RVS - V_{r20}$, $(V_{r20} = \frac{V_{h20}}{V_{i20}})$. Where $V_{h20}$ is the modal value of the hypo-osmotic volume distribution of samples incubated for 20 min (figure 6), and $V_{i20}$ is the modal value of the iso-osmotic volume distribution after 20 minutes of incubation (PETRUNKINA et al., 2001c).
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Figure 5: Volume distribution curves of frozen-thawed bull sperm under iso-osmotic (a) and hypo-osmotic (b) conditions. Hypo-osmotic distribution curve is shifted to larger volume values compared with the iso-osmotic curve; the modal sperm volume increased from 13.50 fl under iso-osmotic (a) to 19.76 fl under hypo-osmotic conditions (b). The distribution shape is changed; the heterogeneity of response is more strongly pronounced. Vertical lines represent the cursor positions that define the sperm population selected for analysis. Particles with lower volumes (cell debris and noise) and larger volumes (agglutinated sperm) are excluded from analysis.
3.4.3 Evaluation of sperm chromatin status (mf-SCSA)

EVENSON and co-workers (1980) developed the sperm chromatin structure assay as flowcytometric method. TEJADA et al. (1984), KOSOWER et al. (1992) and ACEVEDO et al. (2001) modified the test, in order to make a fluorescence microscopic evaluation possible. The protocol of ACEVEDO et al. (2001) modified by WABERSKI and HELMUS (unpublished) was used in the present study.

3.4.3.1 Preparation of semen smears

In this experiment 90 frozen semen straws from 30 bulls (3 straws /bull) were used. At each attempt, 12-16 fine straws (0.25 ml) were thawed in water bath at 39 °C for 10 seconds. The contents were transferred to a graduated centrifuge tubes and filled up to 2 ml with sodium citrate buffer 2.9 % (6.8 pH), then washed two times by centrifugation at 2700 g (3500 rpm) for 10 minutes at room temp (20 °C). The supernatants were sucked off by means of water-operated vacuum pump and the pellets were resuspended each in 2 ml sodium citrate buffer 2.9 % and vortexed to ensure proper pellet disruption. After the second centrifugation, the supernatants were sucked off and the sperm pellets were separately resuspended each in 100 µl citrate buffer. Two thick semen smears were made by placing 50 µl semen suspension on a special glass slide (Superfrost® plus; company Roth, KARLSRUHE) marked with a solvent-free pin (acid- and alcohol proof). The smears were left at room temperature for approximately 10 minutes for air-drying. The further steps of this protocol were accomplished either at the same day or on the following days.

3.4.3.2 Decondensation of sperm chromatin

The procedures for preparation of the solutions used for decondensation and denaturation of sperm chromatin were carried out under an Outlet (vent) using protective masks and gloves. Decondensation solution must be used within 24 h. For each slide, 2 ml decondensation solution was needed. Per passage, 16 smears could be stained (i.e. 32 ml decondensation solution was needed). For preparation of 32 ml solution, 0.0247 gm 1.4-Dithiothreit (DTT) was weighed out on a laboratory balance.
and transferred to a beaker containing 32 ml sodium citrate 2.9 % buffer (corresponds to 5 mM DTT). The next step, 1 mM Dimethylsulfoxide (DMSO) was diluted with 1.75 mM distilled water. Into a separate test tube 2.272 ml DMSO were added with a syringe (correspondence to 1 mM DMSO; 71 µl / ml) and mixed with 1008 µl bi-distilled water (correspondence to 1.75 mM water; 31.5 µl / ml). After accomplish of heat-produced reaction and clear cooling of the test tube, its contents were added to the beaker containing DTT solution. The slides were kept horizontally on a test tube stand under the Outlet (Vent). Each slide was covered with 2 ml of the work solution (DTT / DMSO) and allowed to stand for 30 minutes. Shortly before ending of the decondensation time, a washing bottle with sodium citrate buffer 2.9 % (6.8 pH), a jar with the same buffer and a beaker were being made available. The remained denaturing solution on the slides were poured off in turn over the beaker, rinsed with the washing bottle and placed for eight minutes in a rinsing jar. Afterwards the slides were taken out, dried on the back with cellulose and placed as perpendicularly as possible on an absorbent material (cellulose), in air for approximately 10 minutes.

3.4.3.3 Acid denaturation

The air-dried semen smears were put in a jar containing 60 ml Carnoy’s Solution (20 ml acetic acid + 40 Methanol, pH value 2) for 100 minutes for acid-denaturation. 15 minutes before the end of denaturation time, a staining jar, and a rinsing jar were put in a refrigerator. Upon completion of the denaturation time, the slides were taken out from the jar and wiped with cellulose and then quickly dried in air.

3.4.3.4 Staining with acridin orange (AO)

The prepared semen smears were placed in a pre-cooled staining jar containing AO staining solution, which consisted of 40 ml citric acid solution + 2.5 ml cooled di-sodium hydrogen phosphate solution + 10 ml AO stock solution (cooled, darkly stored) and stored in a refrigerator for 20 min. After staining, the slides were washed gently with a pre-cooled sod citrate buffer 2.9 % then placed in a washing jar
containing the same buffer. 10 min later the smears were taken out from the washing jar, allowed to dry and maintained in a refrigerator until viewing.

### 3.4.3.5 Evaluation of the stained smears

Evaluation of the stained semen smears was carried out in a darkened room by means of fluorescence microscope using a blue laser (490 nm excitation filter, and 520-nm barrier filter), phase 2 and 200x magnification with a 20 objective lens. Thereby, the wavelength 530 nm light emits green (double strand DNA) and 640 nm light emits red (single strand DNA). A digital camera (Olympus DP 50) was mounted onto the fluorescence microscope and coupled with a computer that processes and downloads the digital image through a program called Software analysis® 3.0 (Soft Imaging System GmbH, MÜNSTER). A series of fields per slide were photographed and saved for later evaluation. For each replicate, AO stained spermatozoa were assessed simultaneously in more than 200 spermatozoa in 10 or more individual fields. The fluorescent characteristics of each cell were noted as green (chromatin stable, double-stranded, acid resistant DNA), red-orange (chromatin unstable, single stranded, denatured DNA), half green-half red (partially denatured DNA), pink or yellow (partially denatured DNA) as shown in figure 6.
3.4.4 Oviductal explant assay (OEA)

The cows and heifers included in this experiment were clinically healthy but of unknown previous reproductive history.

3.4.4.1 Preparation of oviductal explants

Oviducts including isthmus, ampulla, infundibulum, fimbria, small part of the uterotubal junction and mesosalpinx were collected from both cows and mature heifers at the local slaughterhouse in Hannover city. The oviducts were collected within 20-30 min of the animal's death. The uterus and ovaries were examined for anomalies and pathological lesions as well as for pregnancy before disposal of the oviducts. Each oviduct was thoroughly washed with sterile PBS and placed in 100 ml of PBS (pH 7.4) then transported on ice to the laboratory. Upon arrival, the oviducts were thoroughly washed with PBS and then dissected free of the surrounding tissues (mesosalpinx) and straightened as much as possible. The ampullary and isthmic
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Segments were cut into 2-3 pieces in large Petri-dish containing PBS. Each piece was taken with a watchmaker’s forceps (tweezers) and held from the narrow end over a small Petri-dish containing few drops of sperm-TALP medium and gently squeezed along the outside toward the wide end with another watchmaker’s forceps to expel epithelium. At every attempt, both oviducts (right and left) from 3-4 cows / heifers were pooled to avoid the individual cow effect as well as the local hormonal effect. The expelled epithelial tissue sheets were disaggregated into small pieces by one passage through a 25-gauge needle attached to 1-ml insulin syringe and transferred to test tube containing 5 ml sperm-TALP, then allowed to stand for 10 min. After initial sedimentation, the supernatant was removed and 5 ml of fresh sperm-TALP was added to the pellet. The same volume was removed again after 10 min (second sedimentation) and the Oviduct Epithelial Cells (OEC) sheets were resuspended in 0.5 ml sperm-TALP and incubated at 39 °C in a humidified atmosphere containing 5 % CO₂. Within 30 min of disaggregation, the clumps of epithelial cells formed everted vesicles with apical surfaces facing outward, henceforth referred to as ‘‘explants’’ (figure 7a).

Figure 7: a) Bovine sperm bound to oviductal epithelial Explants (Phase contrast microscope 200x); b) Scanning micrograph (5000x), bovine sperm bound to the cilia of bovine Oviduct epithelial cells.
3.4.4.2 Preparation of semen samples

In this experiment 90 ejaculates from 30 bulls (3 ejaculates / bull) were used at 35 attempt days. At each attempt day, 3-4 straws from different bulls were thawed and examined subjectively for motility, then passed through discontinuous percoll gradients as described in m-HOST experiment. Aliquots of the recovered spermatozoa were suspended separately in 0.3-0.5 ml warm sperm-TALP and assessed again for motility (motility of washed spermatozoa) on a microscope stage heated at 38 °C and sperm cell concentration using a haemocytometer.

3.4.4.3 Determination of sperm cell concentration

Estimation of the sperm cell concentration was carried out by means of Thoma cell counting chamber. With a micropipette, 20 µl thawed semen was taken and diluted in 480 µl 10 % sodium chloride (dilution rate = 1:25). A coverslip was placed on the haemocytometer counting slide after wetting supports with saliva to tightly hold the coverslip while loading the sperm. 10 -15 µl of the diluted sperm was allowed to flow under the cover slip on each side of the haemocytometer. After 5 minutes the slide was viewed under a phase contrast microscope using 40x magnifications. On each side of the haemocytometer, the spermatozoa were counted in 5 large squares (4 diagonal and one at the corner). The calculation of the sperm cells density in million per µl was made according to the following formula:

\[
\text{The number of sperm} /1 \mu l = \frac{\text{Number of spermatozoa counted in 10 squares}}{\text{Counted surface area} \times \text{chamber depth} \times \text{dilution rate}}
\]

The haemocytometer chamber is 0.1 mm in depth and the 25 large squares represent an area of 1 mm². The volume above the 25 squares shown is 0.1 µl. Only 10 squares were counted and the dilution rate was 1:25, so a factor of 625 was calculated, by which the sum of the counted spermatozoa was multiplied. The result corresponded to the concentration of the sperm cells per µl solution. The sperm cell concentration was adjusted to $5 \times 10^5 / \text{ml}$. 
3.4.4.4 Co-incubation of spermatozoa with oviductal explants

Both the explants and the prepared semen samples were equilibrated for 10 min at 39 °C in a humidified atmosphere containing 5 % CO₂. Afterwards, 10 µl aliquot was taken from the dense layer of explants and transferred to 50 µl droplet of sperm-TALP in a small Petri dish, then 20 µl semen suspension was added to the droplet and gently mixed, so that the final droplet volume was 80 µl, and the final sperm cell concentration was 1.25 x 10⁶ sperm /ml. After 15 min of co-incubation in CO₂ incubator, the explants were washed free of unbound, loosely attached sperm by drawing them up into a 100-µl micropipette and transferring them into fresh 80 µl sperm-TALP droplets. The washing process was repeated three times to assure that all unbound sperm were removed. The explants with bound sperm were then transferred to pre-warmed slide supported by silicon grease and covered with pre-warmed cover slips, then pressed gently for fixation.

3.4.4.5 Video-microscopy and image analysis

The prepared slide was transferred with the cover slip directed downward to a warm stage (38 °C) inverted microscope (IM35; company Zeiss, JENA / GERMANY) equipped with video camera (Kappa, CF 8/1) also coupled with video recorder (SLV-E 720, VHS; company Sony, Japan) and monitor (WV-3M 1400; Panasonic, JAPAN). Explants on each slide were viewed under 256x magnifications (32 x 8). 2 slides per ejaculate were made and in each slide, 6 fragments (sections) of about 2-3 explants (1-2 fragments / explant) were videotaped. All attempts were successively accomplished and taken up on videocassette, so that the photographs could be evaluated to a later time. Beside the bound sperm cells on each attempt day a scale was videotaped in the respective magnification. Videotaping was completed within 12 min for each slide. For analysis, the videotapes were reviewed to count the number of spermatozoa bound to the side of the oviduct explants facing the camera. For counting out the bound sperm, a foil was put over the image plane of the monitor, then the bound spermatozoa were marked with a water-soluble marker and these markings were counted and documented.
3.4.4.6 Estimation the surface area of the explant

The surface areas of the videotaped explant and its fragments were estimated with the help of an image analysis, computer-assisted, surface area measuring program "Aida" (Mika medical GmbH image analysis version 2.0; Copyright 1992; Rosenheim, GERMANY). The computer was coupled with a monitor and video recorder. The scaling for the respective enlargement had to be stored and the computer program was scaled firstly before the computer could accomplish the computations. The videotaped explants were stored separately in the fixed image in order to be able to mark their contour with the mouse on the monitor.

3.4.4.7 Determination of the binding index (BI)

The number of spermatozoa bound to 0.01 mm$^2$ explant's surface was used as a parameter of sperm-oviduct binding capability and called binding index (BI). The calculating BI was adapted after PETRUNKINA et al. (2001b).

The surface areas of 36 fragments per bull (12 fragments / ejaculate and 3 ejaculates / bull) and their bound sperm numbers were submitted to the Mean procedure of SAS to determine the BI for each ejaculate according to the formula:

$$BI = \frac{\sum N_1+N_2+\ldots\ldots\ldots N_{12}}{\sum S_1+S_2+\ldots\ldots\ldots S_{12}}$$

Where $N_{1-12} =$ the number of bound spermatozoa / fragment and $S_{1-12} =$ the surface areas of the explant’s fragments. The BI for each bull was calculated as the mean value of the binding indices of the three ejaculates.

3.4.5 In vitro fertilization (IVF)

Two experiments were carried out at Institute of animal breeding in Mariensee, the federal research institute for agriculture (FAL). The first experiment was carried out in the period between April / 2002 and September / 2002 to investigate the relationship between percentage of spermatozoa with unstable chromatin and the IVF results (cleavage- and blastocyst-rate). The second experiment was carried out between February / 2003 and May / 2003 to investigate the relationship between the ability of
Materials and Methods

spermatozoa to bind to oviductal epithelium and \textit{in vitro} fertility (cleavage- and blastocyst-rate). The procedure that described by WRENZYCKI (1995) with some modifications was used.

In the first experiment, 16 straws from 4 different bulls (4 straws per bull) and 5 straws from one bull, altogether 21 straws from five bulls were examined on 7 attempt days. On each attempt day, three straws from three different bulls were examined. Per straw approximately 55-60 oocytes were used, so that 1217 oocytes were used in the entire experiment. The 5 bulls were divided into 2 groups, the first group include two bulls with relatively low mf-SCSA values ($3.6 \pm 0.7$), and the other three bulls (group II) had relatively high mf-SCSA values ($7.6 \pm 0.4$) (table 5).

In the second experiment 20 straws from four bulls (5 straws per bull) and 12 straws from 2 bulls (6 straws per bulls), altogether 32 straws of the six bulls were examined on 10 attempt days. On each attempt day 3-4 straws of the six bulls were examined. Per straw approximately 50-60 oocytes were used, so that 1899 oocytes were used in the entire experiment. The six bulls in this experiment were divided into two groups. The first group included three bulls with relatively high binding indexes ($19.9 \pm 2.4$) and the second group include bulls with relatively low binding indices ($10.4 \pm 0.5$) as shown in table 8.

3.4.5.1 Collection of ovaries

Ovaries were recovered 20 min after slaughtering from cows and heifers at the slaughterhouse in LÜBBECKE city. The animals were mostly of Holstein origin, whose age and medical history were unknown. No selection with respect to the stage of oestrous cycle was done. However, ovaries from cows with uterine pathology such as pyometra, or ovarian cysts were not collected. Ovaries were collected on fat (mesentery) in an insulated flask (thermos bottle) and being transported to the laboratory within two hours. Upon arrival, the ovaries were washed 2-3 times with warm ($30 \, ^\circ$C) PBS medium before the slicing began.
3.4.5.2 Recovery of oocytes

The oocytes were recovered from the ovaries by the slicing method. The slicing units consist of 6-8 razor blades (0.15 mm, Romi, Solingen, GERMANY) which joined together in a metal skeleton. The slicing device cuts the surface of the ovaries in various dimensions. Ovaries were held in large Petri dish and fixed with artery forceps in PBS medium supplemented with 2 IU heparin (0.0056 g / 500 ml) and 0.1 % BSA. The slicing was made in different dimensions with about 3 mm depth. Following slicing, the resulting fluid was passed through a fine sieve into a glass beaker and allowed to stand ~15 min for sedimentation of cumulus oocyte complexes. The supernatant was removed by means of a water-operated vacuum pump and the sediment re-suspended in about 100 ml PBS (with heparin) was transferred to 15 ml centrifuge tubes (Greiner, NÜRTINGEN, GERMANY) and then the sediment was removed and diluted with fresh PBS (with heparin) medium in 60 mm plastic dishes (Greiner GmbH, NÜRTINGEN, GERMANY) before being viewed under a stereomicroscope. Only class I, i.e. oocytes with a homogeneous evenly granulated cytoplasm possessing at least three layers of compact cumulus cells and class II, i.e. oocytes with fewer than three layers of cumulus cells or partially denuded but also with a homogeneous evenly granulated cytoplasm were selected and transferred to warm collection medium (TCM-air) in small Petri dish on a warm (38 °C) plate. The oocytes were transferred to the maturation medium. Oocytes with degenerated cytoplasm or surrounded by expanded, degenerated, dark looking cumulus cells, were not used in the present study (figure 8).
3.4.5.3 In vitro maturation (IVM)

On the day of use, TCM199 medium was supplemented with 10 % BSA and pyruvate (2.2 mg / 100 ml) to produce washing medium (TCM-pure). A portion of this medium (975 µl) was supplemented with 25 µl Suigonan® (One dose Suigonan® consists of 200 IU hCG and 400 IU eCG, Intervet, TÖNISVORST, GERMANY) to serve as maturation medium. In a medium size culture dishes, the wash drops were prepared at a rate of 12 drops (100 µl) per dish then covered with silicon oil (Serva, HEIDELBERG, GERMANY). For maturation, four 100-µl droplets were prepared in 35 mm sterile polystyrene culture dishes (Greiner GmbH, NÜRTINGEN, GERMANY), then covered with silicone oil and equilibrated in the same culture environment for one h. The immature oocytes were washed three times in washing drops before being transferred in groups of 20-25 to the maturation drops. Equilibration and incubation were carried out at 39 °C in high humidity atmosphere and 5 % CO₂ in air for 23-24 h.

Figure 8: In vitro bovine cumulus oocyte complex, classes I and II.
3.4.5.4 *In vitro* fertilization (IVF)

Modifications of Tyrode Albumin Lactate Pyruvate (TALP) medium after (PARRISH *et al.*, 1988) were used. Sperm-TALP was employed for swim-up separation of the motile fraction of semen and subsequent washing of sperm. It was supplemented with pyruvate and BSA (A-9647 fraction V, Sigma) on the day of use. The other modification, fert-TALP was used for washing of the IVM oocytes before they were placed into the fertilization drops made from fert-TALP medium. This medium was supplemented with gentamicinsulfat, sodium pyruvate and BSA on the day of use. The IVF media were prepared in double distilled water (Ampuwa®, Fresenius AG) and the pH was adjusted to 7.4 then stored at 4 °C after passing through a 0.22 µm in Ø cellulose sterile filter. The fertilization medium was prepared freshly by supplementing fert-TALP with the capacitation inducing agents consisting of hypotaurine, epinephrine and heparin. Both washing and fertilization media were equilibrated in the culture environment for one h prior to insemination. The *in vitro* matured oocytes were washed three times in washing drops under oil and transferred in groups of 20-25 oocytes to the fertilization droplets. The oocytes were then returned to the incubator for at least 30 min until sperm preparation was accomplished.

3.4.5.5 Preparation of spermatozoa and fertilization

Semen was prepared as described by PARRISH *et al.* (1988). In each attempt three straws from three different bulls were group thawed in water bath at 38 °C for 1 min. For swim-up separation of the motile fraction, the content of each straw (0.25 ml) was layered under 1 ml sperm-TALP supplemented with BSA (A-9647 fraction V, Sigma) and pyruvate in sterile glass held at an angle of 45°. The motility of the sperm after thawing was determined under a phase contrast microscope (200x). After one h of incubation at 39 °C under 5 % CO₂ in air, 850 µl from the top of the medium was pipette and transferred into a sterile centrifuge tube. Following the addition of 5 ml sperm-TALP medium, the swim-up separated sperm were centrifuged at 350 g (1200 RPM) at 25 °C for 10 min. The sperm pellets were resuspended each in fresh 5 ml of
sperm-TALP medium and centrifuged again. The final sperm pellets were resuspended each to ~200 µl with fert-TALP and incubated for 15 min at 39 °C under 5 % CO₂ in air for capacitation. During this time sperm concentration was determined using a counter slide (Thoma; Superior, Omnilab, GEHRDEN, GERMANY). The concentration was adjusted to 12.5 million sperm per ml using the fertilization medium for dilution. 2 µl aliquots sperm suspension were transferred to each 100 µl fertilization droplet containing ~20 oocytes to give a final sperm concentration of 0.25 million sperm per ml (suboptimal sperm concentration to be able to differentiates among bulls).

3.4.5.6 Removal of cumulus cells

Fertilized oocytes were denuded from the cumulus cells by vortexing (1200 / min) for 4 min in collection medium (TCM-air) followed by gentle pipetting and collection the denuded ova under a stereomicroscope.

3.4.5.7 In vitro culture of embryos (IVC)

On the day of use, the stock solution of synthetic oviductal fluid (SOF medium) was supplemented with Na-Pyruvate, glutamine, Gentamycin, non-essential amino acids, essential amino acids and polyvinyl alcohol (see appendix). This medium was used as wash and culture medium. Prior to use, the wash and culture dishes were equilibrated in the culture environment for one h. About 18 h following fertilization, presumptive zygotes were denuded of cumulus cells, washed three times in 80 µl droplets of washing medium and then transferred in groups of 6-8 zygotes into 30 µl of culture medium. Zygotes were cultured under silicone oil in 5 % CO₂, 5 % O₂ and 90 % N₂ (Air Product, HATTINGEN, GERMANY) in a humidified atmosphere in Modular incubator (ICN Biomedical, Inc., Aurora, No. 615300, OHIO, USA) at 39 °C for 8 days. The culture medium was not replaced during the culture period. Cleavage rate was evaluated under a stereomicroscope at 45× magnification on day 3 by counting the 2 to 8 cell embryos and referred to the whole of the cultivated embryos also the blastocyst rate was determined on day 8. The embryonic stages were
assessed under a stereomicroscope at 45x magnification after denudation and given below according to LINDNER and WRIGHT (1983) as shown in figure 9.

a) In vitro derived bovine 2-4-cell embryo  
b) In vitro derived bovine 4-8-cell embryo  
c) In vitro derived bovine 8-16 cell embryo  
d) In vitro derived bovine morula  
e) In vitro derived bovine expanded blastocyst  
f) In vitro derived bovine hatched blastocyst

**Figure 9: Different embryonic stages after LINDNER and WRIGHT (1983).**
3.5 STATISTICAL ANALYSIS

The computation and the diagrams of the present study were accomplished using the statistics package SAS/STAT (SAS institute Inc., version V8.3 for Windows, Cary, North Carolina, USA) as well as the Excel software (Microsoft office XP, Inc., USA). The data acquisition and organization were carried out with the data base program (dBASE for Windows, version 3.0). Data were not transformed because they passed tests of normality and homogeneity of variances.

3.5.1 Analysis of volumetric parameters

The data recovered from Latex particles calibration were submitted to the linear regression (REG procedure) of SAS to calculate the correction factor. The calculated correction factors were 1.16 for modal values and 1.11 for the mean values of the sperm cell volume under hypo-osmotic conditions. The corrected CASY data were analysed by the general linear models procedure of SAS (GLM, Least Squares Means). Means are reported as least square mean (LSM ± SD) unless stated otherwise. Comparisons were made within the volume distributions obtained from the replicate ejaculates and among individual bulls.

3.5.2 Analysis of the data of mf-SCSA

The evaluation was done by means of the statistics procedure MEANS of the SAS package. For the question, in any degree the mf-SCSA value was connected with the other spermatological parameters, the Pearson's coefficient of correlation was computed with the SAS procedure CORR. In addition, for the classical spermatology parameters, a stepwise multiple regression analysis was accomplished, in order to find out whether one of the variables had a prognostic value for mf-SCSA.

3.5.3 Analysis of the data of oviductal explants assay (OEA)

The average values and the standard deviations of the binding index were separately calculated for each individual bull using the procedure MEANS. These values served
as design fundamentals for further analyses of a total average value over all bulls and for the comparison among individual bulls. For comparison between bulls the 2-factorial analyses of variance (ANOVA) were accomplished using general linear model (GLM procedure). For determination of the relation between binding index and other spermatological parameters, the correlation analysis was accomplished after Pearson by calculation of the correlation coefficient (r) and the associated probability of mistake (p value) using the procedure CORR of SAS.

3.5.4 Analysis of the IVF data

In the first experiment, which performed to investigate the relationship between sperm chromatin status and the IVF results (cleavage and blastocyst rate), the normal distribution of the results was examined. Since no normal distribution was present, no correlation was computed. Subsequently, the samples were divided with low and high mf-SCSA values into two groups and compared with each other using Wilcoxon two-sample test and in t-test.

Concerning the second IVF experiment that performed to study the relation between sperm oviduct binding ability and fertility _in vitro_ the bulls were classified into two groups according to their binding indices, one with relatively high BI and the other with relatively low BI. The data from six bulls included in this experiment were submitted to the GLM procedure, least square means, NPAR1WAY procedure (ANOVA) and Wilcoxon two-sample test to differentiate between two groups of bulls.

3.5.5 Significance levels for the probability of mistake

For the entire study applies the value of $p \leq 0.05$ as a significant limit value for the probability of null hypothesis. A further level of $P \leq 0.001$ was used, which indicates the limit value of a high-significant probability of mistake. All data represented as mean value ± standard deviation (SD).
4 RESULTS

4.1 STANDARD SPERMATOLOGICAL PARAMETERS

4.1.1 Motility parameters

4.1.1.1 Post-thawing subjective motility

The descriptive statistics of the sperm motility parameters for the 30 bulls involved in the present study are listed in table 1. The motility of frozen-thawed semen was evaluated immediately after thawing. Forward- and local-motile as well as non-motile spermatozoa were estimated to the nearest 5 %. The forward motility % ranged from 40 ± 18.1 % to 75 ± 5 %. The overall mean percentage of forward motile spermatozoa was 60.4 ± 8.2 % (mean ± SD). The most of bulls (26 bulls) recorded values higher than 50 % (the minimum required value), while only 4 bulls had values lower than 50 % as shown in figure 10.

Figure 10: Post-thawing subjective progressive forward motility %. (90 ejaculates from 30 bulls; 3 ejaculates / bull).
4.1.1.2 Post thawing motility using cell motion analyser (CMA)

The overall mean percentage of forward motile spermatozoa was 59.2 ± 8.4 % (mean ± SD), with a maximum value of 72.8 % and a minimum one of 34.3 %. It was not significantly different compared with the subjectively estimated value (60.4 ± 8.2 %), as shown in table 1. Only 4 bulls had recorded forward motility values higher than 50 % (figure 11). The average path-, curvilinear- and straight-line velocities of spermatozoa were 62.75 ± 3.9, 112 ± 8.1 and 55.9 ± 4.7 µm / sec respectively (table 1).

![Figure 11: Post-thawing progressive forward motility %, estimated with CMA (90 ejaculates from 30 bulls; 3 ejaculates / bull).](image)
4.1.1.3 Motility of percoll selected spermatozoa

Immediately after centrifugation of the frozen thawed semen samples on discontinuous percoll gradients, the sperm pellets were resuspended in 200-300 ml warm sperm-TALP and incubated at 39 °C for 5 minutes in CO₂ incubator, then subjectively examined for motility. A highly significant (P < 0.0001) difference was obtained between the forward motility percentage before and after percoll washing. There was a rise from 60.4 ± 8.2 % before washing to 74.5 ± 7.5 % (mean ± SD) after washing, with a range from 55.0 % to 88.4 %. All bulls had recorded values higher than 50 % (the minimum required threshold value of forward motility %) as shown in figure.12.

Figure 12: Post-thawing subjective progressive forward motility % of percoll selected spermatozoa (90 ejaculates from 30 bulls; 3 ejaculates / bull).
<table>
<thead>
<tr>
<th>Item</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subj-f</td>
<td>60.4</td>
<td>8.2</td>
<td>40.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Subj-l</td>
<td>8.9</td>
<td>2.5</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Subj-i</td>
<td>30.7</td>
<td>10.6</td>
<td>15.0</td>
<td>85.0</td>
</tr>
<tr>
<td>Perc-f</td>
<td>74.5</td>
<td>7.5</td>
<td>55.0</td>
<td>88.4</td>
</tr>
<tr>
<td>Perc-l</td>
<td>9.4</td>
<td>2.3</td>
<td>5.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Perc-i</td>
<td>16.1</td>
<td>7.8</td>
<td>3.4</td>
<td>35.0</td>
</tr>
<tr>
<td>CMA-f</td>
<td>59.2</td>
<td>8.4</td>
<td>34.3</td>
<td>72.8</td>
</tr>
<tr>
<td>CMA-l</td>
<td>8.1</td>
<td>2.9</td>
<td>1.0</td>
<td>16.0</td>
</tr>
<tr>
<td>CMA-i</td>
<td>32.7</td>
<td>9.4</td>
<td>17.1</td>
<td>61.1</td>
</tr>
<tr>
<td>VAP</td>
<td>62.75</td>
<td>3.9</td>
<td>47.2</td>
<td>62.4</td>
</tr>
<tr>
<td>VCL</td>
<td>112.0</td>
<td>8.1</td>
<td>87.1</td>
<td>125.4</td>
</tr>
<tr>
<td>VSL</td>
<td>55.9</td>
<td>4.7</td>
<td>33.8</td>
<td>65.5</td>
</tr>
</tbody>
</table>

Table 1: Mean, minimum and maximum values of sperm motility parameters (n = 90 ejaculates from 30 bulls; 3 ejaculates / bull).

Subj-f: Forward motile Spermatozoa (roughly estimated %)
Subj-l: Local-motile spermatozoa (roughly estimated %)
Subj-i: Immotile spermatozoa (roughly estimated %)
Perc-f: Forward motile % of percoll washed spermatozoa (roughly estimated)
Perc-l: Local-motile % of percoll washed spermatozoa (Roughly estimated)
Perc-i: Immotile % of percoll washed spermatozoa (roughly estimated)
CMA-f: Forward motile Spermatozoa (CMA-determined %)
CMA-l: Local-motile spermatozoa (CMA-determined %)
CMA-i: Immotile spermatozoa (CMA-determined %)
VAP: Average path velocity of spermatozoa (µm/sec.)
VCL: Curvilinear velocity of spermatozoa (µm/sec.)
VSL: Straight-line velocity of spermatozoa (µm/s)
CMA: Cell motion analyzer
SD: Standard deviation
4.1.2 Morphological abnormalities of spermatozoa

Spermatozoa with abnormal morphology were classified into two categories. Category 1) HEAD: including primary- and secondary head abnormalities as well as acrosomal abnormalities and category 2) MAS (morphologically altered spermatozoa): including head abnormalities and other abnormalities such as midpiece- and tail abnormalities.

The overall mean percentage of head abnormalities was 30.4 ± 6.6 % (mean ± SD) with a maximum value of 43 % and a minimum one of 21.2 %. Concerning morphologically altered spermatozoa (MAS), the overall mean was 37.4 ± 7.6 % (mean ± SD) and ranged from; 25.4 % to 51.9 % (table 2). All bulls had recorded values less than 50 % except 2 bulls (figure 13).

<table>
<thead>
<tr>
<th>Item</th>
<th>$\bar{x}$</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prim-head</td>
<td>0.8</td>
<td>0.7</td>
<td>0.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Acrosome</td>
<td>29.6</td>
<td>7.9</td>
<td>15.6</td>
<td>38.4</td>
</tr>
<tr>
<td>Head</td>
<td>30.4</td>
<td>6.6</td>
<td>21.2</td>
<td>43.0</td>
</tr>
<tr>
<td>Others</td>
<td>6.6</td>
<td>4.1</td>
<td>1.0</td>
<td>26.0</td>
</tr>
<tr>
<td>MAS</td>
<td>37.4</td>
<td>7.6</td>
<td>25.4</td>
<td>51.9</td>
</tr>
<tr>
<td>Alive</td>
<td>65.6</td>
<td>10.8</td>
<td>26.1</td>
<td>80.9</td>
</tr>
<tr>
<td>Dead</td>
<td>34.5</td>
<td>10.8</td>
<td>19.1</td>
<td>73.9</td>
</tr>
</tbody>
</table>

| Prim-head:  | Primary sperm head abnormalities % |
| Acrosome:   | Acrosomal abnormalities %           |
| Head:       | Primary head, secondary head and acrosomal abnormalities |
| Others:     | Sperm tail and midpiece abnormalities |
| MAS:        | Morphologically altered spermatozoa |
| Alive:      | Alive sperm %                       |
| Dead:       | Dead sperm %                        |

Table 2: Morphological parameters and viability of frozen thawed semen (90 ejaculates from 30 bulls; 3 ejaculates per bull).
4.1.3 Relationship between IVF results and standard spermatological parameters

When the data of the bulls that were included in the two IVF experiments (n=11) were submitted to statistical analysis, no significant correlations existed between cleavage rate and any of the standard spermatological parameters. However, significant (P ≤ 0.05) Correlations were recorded between blastocyst rate and alive sperm %, sperm head abnormalities and total sperm cell abnormalities (table 3).
<table>
<thead>
<tr>
<th>Item</th>
<th>Cleavage Rate</th>
<th>Blastocyst Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RVS</strong></td>
<td>r -0.03</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>p 0.94</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>RVD</strong></td>
<td>r -0.13</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>p 0.70</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>Subj-f</strong></td>
<td>r -0.43</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>p 0.21</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>VSL</strong></td>
<td>r -0.36</td>
<td>-0.36</td>
</tr>
<tr>
<td></td>
<td>p 0.29</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>VAP</strong></td>
<td>r -0.02</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>p 0.93</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>VCL</strong></td>
<td>r -0.20</td>
<td>-0.56</td>
</tr>
<tr>
<td></td>
<td>p 0.09</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Alive</strong></td>
<td>r 0.49</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>p 0.14</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Head</strong></td>
<td>r -0.37</td>
<td>-0.67</td>
</tr>
<tr>
<td></td>
<td>p 0.28</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>MAS</strong></td>
<td>r -0.38</td>
<td>-0.68</td>
</tr>
<tr>
<td></td>
<td>p 0.26</td>
<td>0.02</td>
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<table>
<thead>
<tr>
<th>RVS</th>
<th>Modal value of relative volume shift of spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVD</td>
<td>Modal value of regulative volume decrease of spermatozoa</td>
</tr>
<tr>
<td>Subj-f</td>
<td>Forward motile Spermatozoa (roughly estimated %)</td>
</tr>
<tr>
<td>VSL</td>
<td>Straight-line velocity of spermatozoa (µm / sec)</td>
</tr>
<tr>
<td>VAP</td>
<td>Average path velocity of spermatozoa (µm / sec)</td>
</tr>
<tr>
<td>VCL</td>
<td>Curvilinear velocity of spermatozoa (µm / sec)</td>
</tr>
<tr>
<td>Alive</td>
<td>Alive sperm (%)</td>
</tr>
<tr>
<td>HEAD</td>
<td>Sperm head abnormalities including acrosomal abnormalities (%)</td>
</tr>
<tr>
<td>MAS</td>
<td>Morphologically altered spermatozoa (%)</td>
</tr>
</tbody>
</table>

Table 3: Pearson's correlation coefficients and levels of significance between IVF results and some spermatological parameters (n = 11 ejaculate from 11 Bulls). r = correlation coefficient, p = degree of probability.
4.2 ADVANCED SPERMATOLOGICAL PARAMETERS

4.2.1 Viability assessment using LIVE/DEAD® Sperm Viability Kit

The overall mean alive sperm percentage was 65.5 ± 10.8 % (mean ± SD) with a minimum value of 26.1 % and a maximum one of 80.9 % (table 2). Concerning the differences among individual bulls, more than 83 % of bulls (n = 25) showed values between 54.8 % (mean - SD) and 76.32 % (mean + SD), while only 3 bulls showed values less than 54.8 % as shown in figure 14.

![Figure 14: Alive sperm % as determined using LIVE/DEAD® Sperm viability kit. (90 ejaculates from 30 bulls; 3 ejaculates / bull).](image)

4.2.2 Modified hypo-osmotic swelling test (m-HOST)

The descriptive statistics of the sperm cell volumetric parameters are illustrated in table 4. The overall mean of the modal values of sperm cell volume distribution under iso-osmotic conditions remained nearly constant along the entire incubation period.
(13.4 ± 3.6 fl at 5 min vs. 13.8 ± 3.5 fl at 20 min), but increased to 21.3 ± 4.8 fl (mean ± SD) under hypo-osmotic conditions after 5 min of incubation at 38 °C, then decreased to 18.8 ± 4.9 fl (mean ± SD) after 20 min of incubation under hypo-osmotic conditions.

Table 4: Sperm cell volumetric parameters (90 ejaculates from 30 bulls).

<table>
<thead>
<tr>
<th>Item</th>
<th>( \bar{x} )</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vi5m</td>
<td>32.5</td>
<td>2.4</td>
<td>26.4 fl</td>
<td>41.3 fl</td>
</tr>
<tr>
<td>Vh5m</td>
<td>39.1</td>
<td>3.3</td>
<td>32.1 fl</td>
<td>50.1 fl</td>
</tr>
<tr>
<td>Vi20m</td>
<td>32.8</td>
<td>3.1</td>
<td>23.3 fl</td>
<td>41.7 fl</td>
</tr>
<tr>
<td>Vh20m</td>
<td>38.4</td>
<td>3.6</td>
<td>31.2 fl</td>
<td>49.8 fl</td>
</tr>
<tr>
<td>Vr5m</td>
<td>1.2</td>
<td>0.1</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Vr20m</td>
<td>1.2</td>
<td>0.1</td>
<td>0.1</td>
<td>1.6</td>
</tr>
<tr>
<td>RVDm</td>
<td>-0.1</td>
<td>0.2</td>
<td>-0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Vi5</td>
<td>13.4</td>
<td>3.6</td>
<td>9.7 fl</td>
<td>33.0 fl</td>
</tr>
<tr>
<td>Vh5</td>
<td>21.3</td>
<td>4.8</td>
<td>13.1 fl</td>
<td>31.9 fl</td>
</tr>
<tr>
<td>Vi20</td>
<td>13.8</td>
<td>3.5</td>
<td>10.2 fl</td>
<td>29.5 fl</td>
</tr>
<tr>
<td>Vh20</td>
<td>18.8</td>
<td>4.9</td>
<td>11.7 fl</td>
<td>32.2 fl</td>
</tr>
<tr>
<td>RVS</td>
<td>1.7</td>
<td>0.5</td>
<td>0.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Vr20</td>
<td>1.4</td>
<td>0.4</td>
<td>0.7</td>
<td>2.8</td>
</tr>
<tr>
<td>RVD</td>
<td>0.3</td>
<td>0.6</td>
<td>-1.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Vi5m: Mean value of sperm volume under iso-osmotic conditions at 5 min (fl)
Vh5m: Mean value of sperm volume under hypo-osmotic condition at 5 min (fl)
Vi20m: Mean sperm volume under iso-osmotic condition at 20 min (fl)
Vh20m: Mean value of sperm volume under hypo-osmotic condition at 20 min (fl)
Vr5m: Relative shift of mean value of sperm volume after 5 min
Vr20m: Relative shift of mean value of sperm volume after 20 min
RVDm: Regulatory volume decrease (mean value)
Vi5: Modal value of sperm volume under iso-osmotic conditions at 5 min (fl)
Vh5: Modal value of sperm volume under hypo-osmotic condition at 5 min (fl)
Vi20: Modal value of sperm volume under iso-osmotic condition at 20 min (fl)
Vh20: Modal value of sperm volume under hypo-osmotic condition at 20 min (fl)
RVS: Relative volume shift (modal value)
Vr20: Relative shift of modal sperm volume after 20 min
RVD: Regulatory volume decrease (modal value)
fl: Femtoliter

Table 4: Sperm cell volumetric parameters (90 ejaculates from 30 bulls).
4.2.2.1 Relative volume shift (RVS)

The overall mean value of relative sperm volume increase was $1.7 \pm 0$ (mean ± SD) with a minimum value of 0.9 and a maximum one of 2.7. The most of bulls recorded values between 1.4 and 2. Only three bulls had shown values more than 2 (mean + SD), while six bulls showed values less than 1.4 (mean - SD). Additionally, clear significant differences in RVS among bulls were observed as shown in figure 15.

Figure 15: Relative shift of modal sperm cell volume ($n = 90$ ejaculates from 30 bulls; 3 ejaculates per bull). Bulls with different letters have significantly ($p \leq 0.05$) different values.
4.2.2.2 Regulatory volume decrease (RVD)

Concerning the regulative decrease of modal values of sperm volume distribution curve (RVD), significant differences were reported among bulls (figure 16). The overall mean was 0.3 ± 0.6 (mean ± SD) with a minimum value of -1.2 and a maximum value of +1.6. Two bulls recorded values more than 0.7 (mean + one SD), and only three bulls had values less than 0.2 (mean – one SD), while the rest of bulls (18 bulls) recorded values between -0.2 and +0.7 (figure 16).

Figure 16: Regulatory volume decrease (RVD). (n = 90 ejaculates from 30 bulls; 3 ejaculates per bull). Bulls with different letters have significantly (p ≤ 0.05) different values.
4.2.3 Modified florescence microscopical SCSA (mf-SCSA)

The overall mean percentage of the spermatozoa with unstable chromatin was 4.6 ± 1.4 % (mean ± SD), with a minimum value of 3.1 % and a maximum value of 7.9 %. About 70 % of the bulls (n = 21) recorded values less than 5 % and 30 % (n = 9) showed higher values (> 5 %) of spermatozoa with unstable chromatin as shown in figure 17.

![Figure 17: Percentage of spermatozoa with unstable Chromatin. (n = 90 ejaculates from 30 bulls, 3 ejaculates per bull).](image)

4.2.3.1 Relationship between sperm chromatin status and IVF results

This experiment was carried to investigate the relationship between sperm chromatin status and the results of in vitro fertilization (cleavage- and blastocyst-rate). Five ejaculates from five different bulls were used. Two bulls (group I) had relatively low mf-SCSA values (3.6 ± 0.7), and the other three bulls (group II) had relatively high mf-SCSA values (7.6 ± 0.4) as shown in table 5. The selected bulls had apparently normal classical spermatological parameters. Two bulls in the group 2 with a relatively high abnormal sperm % and low alive sperm % were included in this
experiment because there were no more bulls in this area (sperm with relatively high instable chromatin). The standard spermatological parameters of the 5 bulls included in this experiment are illustrated in table 5.

<table>
<thead>
<tr>
<th>Item</th>
<th>Bulls with low % unstable sperm chromatin (group 1)</th>
<th>Bulls with high % unstable sperm chromatin (group 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull No.</td>
<td>8 10  x</td>
<td>9 20 11 x</td>
</tr>
<tr>
<td>Chrom</td>
<td>4.1 3.1 3.6 ± 0.7</td>
<td>7.2 7.6 7.9 7.6 ± 0.4</td>
</tr>
<tr>
<td>Subj-f</td>
<td>75.0 66.7 70.9 ± 5.9</td>
<td>60 51.7 60 57.3 ± 4.8</td>
</tr>
<tr>
<td>Perc-f</td>
<td>88.4 81.7 85.1 ± 4.8</td>
<td>75 66.7 71.7 71.2 ± 4.2</td>
</tr>
<tr>
<td>Alive</td>
<td>80.9 66.0 73.5 ± 10.6</td>
<td>47.6 74.2 65.5 62.5 ± 13.5</td>
</tr>
<tr>
<td>Head</td>
<td>20.4 26.5 23.5 ± 4.4</td>
<td>32.4 37.2 23 30.9 ± 7.3</td>
</tr>
<tr>
<td>MAS</td>
<td>25.4 29.5 27.5 ± 2.9</td>
<td>39.4 48.9 33.5 40.6 ± 7.8</td>
</tr>
<tr>
<td>RVS</td>
<td>1.24 1.64 1.5 ± 0.3</td>
<td>1.5 1.7 1.44 1.6 ± 0.14</td>
</tr>
<tr>
<td>RVD</td>
<td>-0.37 0.26 -0.05 ± 0.4</td>
<td>0.32 0.54 0.02 0.29 ± 0.26</td>
</tr>
<tr>
<td>VAP</td>
<td>66.0 66.5 66.3 ± 0.4</td>
<td>68.6 61.0 63.1 64.3 ±4.0</td>
</tr>
<tr>
<td>VSL</td>
<td>57.7 58.4 58.1 ± 0.5</td>
<td>60.3 56.3 55.5 57.4 ± 2.6</td>
</tr>
</tbody>
</table>

Data are the mean value of three ejaculates per bull

<table>
<thead>
<tr>
<th>Chrom</th>
<th>Percentage of spermatozoa with unstable chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subj-f:</td>
<td>Forward motile Spermatozoa (roughly estimated %)</td>
</tr>
<tr>
<td>Perc-f:</td>
<td>Forward motile percoll washed spermatozoa (roughly estimated %)</td>
</tr>
<tr>
<td>Alive</td>
<td>Alive sperm (%)</td>
</tr>
<tr>
<td>Head</td>
<td>Sperm head abnormalities including acrosomal abnormalities (%)</td>
</tr>
<tr>
<td>MAS</td>
<td>Morphologically altered spermatozoa (%)</td>
</tr>
<tr>
<td>RVS</td>
<td>Modal value of relative volume shift</td>
</tr>
<tr>
<td>RVD</td>
<td>Modal value of regulative volume decrease</td>
</tr>
<tr>
<td>VAP</td>
<td>Average path velocity of spermatozoa (µm/sec.)</td>
</tr>
<tr>
<td>VSL</td>
<td>Straight-line velocity of spermatozoa (µm/s)</td>
</tr>
</tbody>
</table>

Table 5: The sperm chromatin stability % and other spermatological parameters for two groups of bulls (values are averages of 3 ejaculates per bull (n = 5 bulls).
When the Pearson’s correlation coefficient estimated on the base of the mean values of individual bulls, a significant correlation (p < 0.05) was obtained between mf-SCSA value and cleavage rate, but not with blastocyst rate. Moreover comparison between the two groups of bulls, revealed significance differences in blastocyst rate (P < 0.01) and cleavage rate (P < 0.05) as shown in figure 18 and table 6.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bull No.</th>
<th>Chrom</th>
<th>Oocytes (n)</th>
<th>Cleavage rate %</th>
<th>Blastocyst rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>( \bar{x} )</td>
<td>SD ( SD )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>4.1</td>
<td>291</td>
<td>62.8</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.1</td>
<td>204</td>
<td>62.1</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>7.2</td>
<td>214</td>
<td>40.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>7.9</td>
<td>285</td>
<td>55.8</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7.6</td>
<td>223</td>
<td>56.0</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Table 6: Cleavage- and blastocyst rates of individual bulls in relation to their chromatin instability % (n = 5 bulls).

- Group I: bulls with relatively low unstable sperm chromatin (3.6±0.7%).
- Group II: bulls with relatively high unstable sperm chromatin (7.6±0.4%).
Results

Figure 18: Cleavage- and blastocyst rates in two groups of bulls.  
Group 1: bulls with relatively low instable sperm chromatin (3.6±0.7%).  
Group 2: bulls with relatively high instable sperm chromatin (7.6±0.4%).  
a, b : significantly different values (p < 0.05).  
c, d : significantly different values (p < 0.01).

4.3 OVIDUCTAL EXPLANT ASSAY (OEA)

Bull sperm attached rapidly to the oviductal explants. Despite gentle swirling after sperm addition and before videotaping, attached spermatozoa were not evenly distributed over the surfaces of the oviductal explants. They were spaced closely in some areas, sparsely in others, and absent in a few areas. Sperm appeared to adhere to the oviductal explants by rostral surface of the head and most of them remained motile (98%). Viability of the oviductal explants was judged by vigorous ciliary’s activity of ciliated cells. The ciliary’s beats were strong and were apparent during the entire experiment. The overall mean of the binding indices was 15.1 ± 2.9 sperm / 0.01 mm² (mean ± SD), with a range from 10.0 to 22.59.
4.3.1 Differences among individual bulls

Statistical analysis of the data from 30 bulls (3 ejaculates per bull) revealed a significant ($P = 0.01$) effect of individual bulls on the ability of sperm to bind to the oviduct epithelial explants. Moreover, significant ($P \leq 0.05$) differences were observed among semen samples from different individual bulls in their binding indices. More than 76% of bulls (23 bulls) recorded binding indices between 12.2 and 18.0, while only 10% of bulls (3 bulls) recorded values more than 18.0 (mean + one SD), and 13.4% of bulls (4 bulls) recorded values less than 12.2 (mean – one SD) as shown in figure 19.

Figure 19: Sperm-oviduct explant Binding Indices of individual Bulls. (90 ejaculates from 30 bulls). Bulls with different letters have significant different binding indices.
4.3.2 Relationship between sperm-oviduct binding ability and IVF results

This experiment was carried out to investigate the relationship between sperm oviduct binding ability and fertility in vitro. Six bulls were selected for this experiment, three of them with relatively high BI and the others with relatively low BI. The IVF parameters (cleavage and blastocyst rates) and BI of the selected bulls are listed in table 7.

Due to the small number of the bulls used in this experiment, no correlations between IVF results and BI were performed. However, comparison between the 2 groups using Wilcoxon two-sample test revealed a significant difference between the 2 groups in blastocyst rate (P = 0.004), on the other hand, no significant (P = 0.48) difference between the two groups in cleavage rate was recorded (figure 20). Concerning the other spermatological parameters, no significant differences were recorded between the 2 groups (table 8).

<table>
<thead>
<tr>
<th>Bull No</th>
<th>BI</th>
<th>Group</th>
<th>Oocytes (n)</th>
<th>Cleavage rate (%)</th>
<th>Blastocyst rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\bar{x})</td>
<td>SD</td>
</tr>
<tr>
<td>1 19.3</td>
<td>293</td>
<td>1 (high BI)</td>
<td>61.5</td>
<td>16.9</td>
<td>40.7</td>
</tr>
<tr>
<td>3 17.9</td>
<td>312</td>
<td></td>
<td>63.7</td>
<td>10.7</td>
<td>47.7</td>
</tr>
<tr>
<td>15 22.5</td>
<td>309</td>
<td></td>
<td>53.3</td>
<td>3.1</td>
<td>51.0</td>
</tr>
<tr>
<td>25 10.0</td>
<td>330</td>
<td>2 (low BI)</td>
<td>53.5</td>
<td>13.3</td>
<td>34.7</td>
</tr>
<tr>
<td>26 10.2</td>
<td>346</td>
<td></td>
<td>70.1</td>
<td>6.9</td>
<td>60.6</td>
</tr>
<tr>
<td>27 10.9</td>
<td>309</td>
<td></td>
<td>64.5</td>
<td>11.6</td>
<td>47.9</td>
</tr>
</tbody>
</table>

Table 7: Cleavage and blastocyst rates of two groups Bulls with low and high binding indices (n= 6 bulls).
Table 8: Sperm oviduct BI and spermatological parameters of two groups of bulls (n = 6). Values with different letters in the same row are significantly different.

* = P ≤ 0.05; ** = P ≤ 0.001.
4.4 CORRELATION MATRIX AMONG SPERMATOLOGICAL PARAMETERS

Data of the 30 bulls included in this study were submitted to statistical analysis to investigate the relationship between sperm oviduct interaction and both sperm cell membrane functional integrity (m-HOST) and sperm chromatin status (mf-SCSA) as well as other standard spermatological parameters. Pearson’s Correlation matrix of all spermatological parameters is presented in table 9.
4.4.1 Relationship between sperm-oviduct binding ability (Bl) and other spermatological parameters

1) Traditional spermatological parameters

No significant correlations were found between sperm oviduct binding capacity and any of the conventional spermatological parameters. A highly significant correlation ($P = 0.0001$) was recorded between Bl and progressive forward motility of Percoll washed spermatozoa.

2) Sperm membrane functional activity (m-HOST)

A significant positive correlation ($P < 0.05$) was recorded between binding ability of sperm to oviduct epithelial explants and all sperm volumetric parameters calculated from CASY1. The Pearson’s correlation coefficient was +0.50 for relative shift of modal sperm cell volume (RVS) and +0.42 for regulative sperm cell volume decrease (RVD).

3) Percentage of spermatozoa with unstable chromatin (mf-SCSA)

A significant ($P = 0.02, r = -0.40$) negative correlation was recorded between sperm oviduct binding index and percentage of spermatozoa with unstable chromatin.

4.4.2 Relationship between sperm chromatin status (mf-SCSA) and other spermatological parameters

1) Conventional spermatological parameters

There was no significant correlation between percentage of spermatozoa with unstable chromatin and any of conventional spermatological parameters. A significant negative ($P = 0.03$) correlation was observed between mf-SCSA values and forward motility % of percoll-selected spermatozoa.

2) Sperm membrane functional activity (m-HOST)

A significant negative correlation was observed between percentage of spermatozoa with unstable chromatin and relative volume shift (RVS) of spermatozoa ($p = 0.02$).
Meanwhile, no significant correlation was recorded between mf-SCSA value (percentage of spermatozoa with unstable chromatin) and regulatory volume decrease (RVD).

4.4.3 Relationship between sperm membrane functional status (m-HOST) and standard spermatological parameters

There was no significant correlation between sperm volumetric parameters and any of conventional spermatological parameters, except for progressive forward motility % of Percoll washed spermatozoa, where a significant (P= 0.03) negative correlation was detected.
<table>
<thead>
<tr>
<th>Item</th>
<th>BI</th>
<th>RVS</th>
<th>RVD</th>
<th>Subj-f</th>
<th>Perc-f</th>
<th>Alive</th>
<th>Head</th>
<th>MAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>RVS</td>
<td>r</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>RVD</td>
<td>r</td>
<td>0.42</td>
<td>0.78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.02</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subj-f</td>
<td>r</td>
<td>0.20</td>
<td>0.19</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.30</td>
<td>0.33</td>
<td>0.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perc-f</td>
<td>r</td>
<td>0.70</td>
<td>0.40</td>
<td>0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.001</td>
<td>0.03</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>r</td>
<td>0.23</td>
<td>0.25</td>
<td>0.01</td>
<td>0.27</td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.21</td>
<td>0.17</td>
<td>0.94</td>
<td>0.16</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>r</td>
<td>-0.02</td>
<td>0.14</td>
<td>0.04</td>
<td>-0.77</td>
<td>-0.40</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.87</td>
<td>0.45</td>
<td>0.81</td>
<td>.0001</td>
<td>0.01</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>MAS</td>
<td>r</td>
<td>-0.06</td>
<td>0.04</td>
<td>-0.01</td>
<td>-0.77</td>
<td>-0.45</td>
<td>-0.12</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.73</td>
<td>0.80</td>
<td>0.93</td>
<td>.0001</td>
<td>0.01</td>
<td>0.49</td>
<td>.0001</td>
</tr>
<tr>
<td>chrom</td>
<td>r</td>
<td>-0.40</td>
<td>-0.41</td>
<td>-0.15</td>
<td>-0.12</td>
<td>-0.39</td>
<td>-0.16</td>
<td>-0.13</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.02</td>
<td>0.02</td>
<td>0.40</td>
<td>0.50</td>
<td>0.03</td>
<td>0.37</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 9: Pearson’s correlation matrix of both standard and advanced spermatological parameters.
5 DISCUSSION

So far sperm oviduct interaction has been studied in many animal species using *in vitro* coculture of spermatozoa with oviduct epithelial cells. These studies were mostly focused on the mechanisms and biological aspects of sperm oviduct binding. The present in vitro studies were conducted to further characterize the functional aspects of the interaction between frozen-thawed bull spermatozoa and the oviductal epithelium. Differences in the initial binding capacity of frozen-thawed sperm to oviductal epithelium using the oviduct explant assay (OEA) were studied in repeated ejaculates from 30 bulls. Additionally, the relationships between sperm-oviductal epithelium binding capacity, membrane functional integrity and chromatin stability as well as their relation to fertility in vitro were investigated.

5.1 OVIDUCT EXPLANT ASSAY (OEA)

Although several studies have shown that adhesion to the oviductal epithelium allows the selection of spermatozoa characterized by intact acrosome (GUALTIERI and TALEVI, 2000), an uncapacitated status (THOMAS *et al*., 1995*a*; LEFEBVRE and SUAREZ 1996; FAZELI *et al*., 1999), low internal free calcium content and reduced tyrosine phosphorylation of membrane proteins (DOBRINSKI *et al*., 1996; PETRUNKINA *et al*., 2001*a*), superior morphology (THOMAS *et al*., 1994*b*) and normal chromatin structure (ELLINGTON *et al*., 1999*a*), until now it is difficult to determine whether these features of spermatozoa are associated with high fertilization competence. It has been emphasized that for investigation of the sperm-oviduct interaction, the *in vitro* conditions should mimic the *in vivo* situation as closely as possible. Current methods for investigating the sperm-oviduct epithelial cell interaction rely on the use of *in vitro* models, either oviductal epithelial explants (pigs: SUAREZ *et al*., 1991; horse: THOMAS *et al*., 1994*a*; hamster: DE MOTT *et al*., 1995; bovine: LEFEBVRE *et al*., 1995) or oviductal epithelial cells monolayers (bovine: POLLARD *et al*., 1991 GUALTIERI and TALEVI, 2000; horse: DOBRINSKI *et al*., 1996; THOMAS *et al*., 1995*a*; human: ELLINGTON *et al*., 1999*a*; porcine: FAZELI *et al*., 1999*a*).
al., 1999). Oviductal explants were used in this study, as it has been found that explants mimic the *in vivo* situation and maintain most of the morphological characteristics of oviductal epithelium (LEFEBVRE et al. 1995). Moreover the oviductal explants were found to be physiologically the more responsive model than oviduct epithelial cell monolayers (BUREAU et al., 2002).

In the current study the oviductal explants were obtained from both isthmus and ampulla, since no significant regional effect of these two origins of explants could be detected *in vitro* (pig: SUAREZ et al., 1991; PETRUNKINA et al., 2001b; cattle: LEFEBVRE et al., 1995; hamster: SMITH and YANAGIMACHI, 1991).

The spermatozoa were co-incubated with oviductal epithelial explants for 15 min and this incubation period resulted in detection of a representative number of spermatozoa that attached to oviductal epithelial explants. GREEN et al. (2001) reported that co-incubation periods < 15 min resulted in minimal binding of sperm to oviductal epithelial cells, whereas only small increases in binding capacity were detected after periods > 15 min.

We observed that spermatozoa attached to the epithelial surface of the explants within minutes and most of them (> 95 %) remained motile, as they could be identified by their long rapidly beating tails. Spermatozoa were found to bind mostly by their heads and preferentially bind to the cilia of the oviductal cells and were not evenly distributed over the surface. They were spaced closely in some areas, and sparsely in others and were absent in a few areas, as has been observed previously in dairy cattle (HUNTER et al., 1991; POLLARD et al., 1991), equine (THOMAS et al., 1994a; b) and swine (RAYCHOUHDHURY and SUAREZ, 1991; SUAREZ et al., 1991). Our observations are inconsistent with THOMAS et al. (1994a) who reported that stallion sperm seemed to bind equally as well to non-ciliated oviductal epithelial cells and with HUNTER et al. (1991) who found that bull sperm attached to microvilli of non-ciliated secretory oviductal cells in scanning electron micrographs of oviducts of naturally mated cows. Accordingly, SMITH and YANAGIMACHI (1990) stated that hamster spermatozoa were found to bind to both ciliated and secretory cells in the oviductal epithelium.
Differences among bulls in their capacity to establish a sperm reservoir probably exist after mating or insemination, but it is difficult to detect these differences after collection of oviducts because the number of sperm cells reported to reach the oviduct in vivo has differed considerably among studies and within experiments (SUAREZ et al., 1997; PARKER et al., 1975; MBURU et al., 1996). After using the above discussed model assay (OEA), clear significant differences were detected among individual semen samples in their initial binding capacity to the oviductal epithelium, indicating the utility and suitability of this assay as a new semen evaluation method. The individual differences of sperm binding capacity to the oviductal epithelium has been reported also in vitro for stallion (THOMAS and BALL, 1996), boar (PETRUNKINA et al., 2001b) and bull (DE PAUW et al., 2002) spermatozoa. These differences among bulls indicate a selective function of the oviductal epithelium and the preferential initial binding of bull sperm to oviductal epithelium in vitro might represent a mechanism for selecting functionally competent sperm. The nature of the selective mechanism is not clear and may rely on the differences in the expression of binding sites on the spermatozoa. At the time of ejaculation, a coat of seminal plasma proteins becomes associated with the sperm surface (TÖPFER-PETERSEN, 1999) and thus such proteins are likely to enter the oviductal sperm reservoir where they could contribute to sperm–oviduct binding.

In the present study, the overall mean number of spermatozoa that bound to 0.01 mm$^2$ explants was relatively lower than that reported in other studies (swine: GREEN et al., 2001; cattle: DE PAUW et al., 2002). These differences may be either due to different experimental conditions and/or to the method of data analysis.

5.1.1 Relationship between sperm-oviductal epithelium binding capacity and chromatin stability

In the present study, a clear significant negative correlation was recorded between the number of spermatozoa that bound to oviductal explants (BI) and the percentage of spermatozoa with unstable chromatin in the ejaculate. This indicates that spermatozoa with stable chromatin were preferentially selected by the oviductal epithelium. These results support our previous findings (WABERSKI et al., 2003a),
as the spermatozoa with stable chromatin in the Percoll selected sperm subpopulation were found to preferentially bind to oviductal epithelium. Our results are in agreement with those of ELLINGTON et al. (1999a, 2000) who observed a selective binding of spermatozoa with stable chromatin to the oviductal epithelium in human and bull semen after co-incubation with oviductal epithelial cell monolayers. The selection mechanism in the female genital tract concerning the sperm chromatin status seems to exist independently for sperm motility and sperm morphology, because no correlations were detected between sperm chromatin status and classical spermatological parameters.

It has been reported that co-culture of human sperm with bovine oviduct epithelial cells reduces sperm chromatin structural changes that were observed during culture in media alone (ELLINGTON et al., 1998a). The mechanisms by which the spermatozoa with stable chromatin selectively bind to oviduct epithelial cells and the prolongation of sperm chromatin stabilization when spermatozoa co-cultured with oviductal epithelial cells are not fully understood. The maturation status of spermatozoa was found to play an important role in the ability of spermatozoa to bind to oviductal epithelium as it has been reported that epididymal spermatozoa and spermatozoa with protoplasmic droplets (immature spermatozoa) were poorly bound to oviductal epithelium compared with ejaculated (mature) boar spermatozoa (PETRUNKINA et al., 2001b; MAGNUS, 2002; WABERSKI et al., 2003b). Furthermore, the immature epididymal spermatozoa are characterized by not well condensed (unstable) chromatin (BEDFORD et al., 1973; KOSOWER et al., 1992; LÖHMER 2003). The results of the present study may indicate that a certain stage of maturation is needed to complete the binding capacity of bull spermatozoa to oviductal epithelium.

The freeze-thawing procedures can affect both sperm chromatin status and sperm oviductal epithelium binding capacity, since it has been reported that cryopreservation induces sub-lethal cellular damages in sperm including: partial capacitation, loss of chromatin integrity, ultra-structural damage and accumulation of reactive oxygen species (MAHADEVAN and TROUNSON, 1984; ALVAREZ and STOREY, 1992; CORMIER et al., 1997; ELLINGTON et al., 1998a). Moreover
ELLINGTON et al. (1999b) reported that human sperm attach to bovine OEC *in vitro* in a dose-dependent manner. Additionally, binding capacity was decreased for cryopreserved percoll-washed sperm although the motility did not differ (ELLINGTON et al., 1999b). Furthermore, HAMMADEH et al. (1999) concluded that freeze-thawing processes cause significant damage to chromatin condensation status, morphology and membrane integrity of spermatozoa in both fertile and infertile men.

5.1.2 Relationship between sperm-oviductal epithelium binding capacity and sperm volumetric parameters

To our knowledge this is the first trial to investigate the direct relationship between the capacity of frozen-thawed bull spermatozoa to bind to oviductal epithelium (tested in OEA) and sperm volume regulation ability (tested in m-HOST). Significant positive correlations were obtained between the binding index (BI) and both RVS and RVD. Sperm samples with high binding index responded well to hypo-osmotic stress (high relative volume shift) and were characterised also by a high rate of regulatory volume decrease when incubated for 20 min under hypo-osmotic conditions. These results revealed that the functional integrity of sperm membrane plays a basic role in the sperm-oviductal epithelium binding ability. In accordance, the results of UCHIDA et al. (1992) revealed better in vivo ability of normal HOST human spermatozoa to interact with the female reproductive tract.

This relationship could be discussed on the base of changes that occur during cryostorage and capacitation processes, which may have an effect on both sperm volume regulation ability and sperm-oviductal epithelium binding ability. Changes in the plasma membrane after freeze–thawing procedures or cooling to low temperatures have been reported to be similar to those that occur during capacitation (WATSON, 1995; FULLER and WHITTINGHAM, 1997; GILLAN et al., 1997) and the capacitation process was found to destabilize the sperm plasma membrane (YANAGIMACHI, 1994; LANGLAIS and ROBERTS, 1985). Furthermore, during the process of cryopreservation sperm are exposed to considerable changes in osmolality, as well as mechanical cell injury could occur leading to intracellular or extracellular ice crystal formation and signs of osmotic damage (WATSON, 1995;
Furthermore it has been reported that freezing causes extensive physical-chemical damage to the extracellular and intracellular membranes of the sperm that are attributable to changes in the lipid phase transition and/or increased lipid peroxidation (ALVAREZ and STOREY, 1992; 1993; MOSSAD et al., 1994) during cooling or after thawing. HAMMADEH et al. (1999) reported that cryoinjury to the plasma membrane in the tail region of human spermatozoa as assessed by HOST was significantly higher after thawing in comparison to before freezing. Moreover ELLINGTON et al. (1999b) reported that attachment of human spermatozoa was decreased for cryopreserved sperm versus fresh sperm in a manner separate from any differences in sperm motility. Furthermore DOBRINSKI et al. (1995) reported that cryopreservation reduces the ability of equine spermatozoa to attach to oviductal epithelial cell monolayers in vitro. Accordingly, more fresh sperm were found to attach to OEC than that recorded for frozen-thawed semen in cattle (GOLDMAN et al., 1998).

Concerning the sperm capacitation process and its effect on sperm volume regulation and sperm oviduct binding, PETRUNKINA et al., (2004) reported that during capacitation of boar sperm, a decrease in swelling level and disturbance of the regulatory volume function were observed. These findings conform to those reported previously (PETRUNKINA et al., 2000; PETRUNKINA and TÖPFER-PETERSEN 2000), where a decrease in relative volume swelling during incubation was observed for both ejaculated and epididymal boar sperm exposed to capacitating conditions. It has also been reported that uncapacitated bull (LEFEBVRE and SUAREZ, 1996) and boar (FAZELI et al., 1999) spermatozoa were selectively and preferentially bound to oviductal epithelium.

5.1.3 Relationship between sperm oviduct binding capacity and fertility in vitro

In the present study, a clear significant difference was detected in the blastocyst rate between the two selected groups of bulls; the first one was with relatively high binding indices showed high blastocyst rate and the second one was with relatively low binding indices showed low blastocyst rate. The individual bulls included in this
experiment had normal standard spermatological parameters (Table 7). So the significant difference in the blastocyst rate may rely only on the difference in binding indices, not on the other sperm parameters.

The interpretation of this relationship could rely on the fact that spermatozoa, which can bind to oviduct explants, are characterized by an uncapacitated status (LEFEBVRE and SUAREZ, 1996), an intact acrosome (GUALTIERI and TALEVI, 2000), a superior morphology (THOMAS et al., 1994b) and a normal chromatin structure (ELLINGTON et al., 1999a).

THUNDATHIL et al. (1999) demonstrated that the proportion of uncapacitated spermatozoa present in frozen-thawed bull semen varies among bulls and more important that the presence of uncapacitated spermatozoa is positively correlated with fertility. Uncapacitated spermatozoa have an advantage compared to capacitated spermatozoa during their transit to the site of fertilization in the oviduct, because they are more likely to survive. If capacitation occurred before spermatozoa reached the oviduct, the sperm population available for fertilization may be reduced causing an adverse effect on fertilization. When the percentage of uncapacitated spermatozoa in a sperm sample is high, more spermatozoa are able to bind to oviduct explants, which may result in a higher fertility rate. Moreover, capacitation is known to destabilize the sperm plasma membrane (YANAGIMACHI, 1994; LANGLAIS and ROBERTS, 1985) and thus reduce the lifespan of spermatozoa (WATSON, 1995). In a previous study which was carried out on cattle, DE PAUW et al. (2002) demonstrated that the number of spermatozoa bound to oviduct epithelial explants after 24 h of co-incubation was positively correlated with the non return rate (NNR) when the membrane integrity of the initial sperm sample more than 60 %.

5.1.4 Relationship between sperm–oviduct binding ability and standard spermatological parameters

The results of the present study revealed no significant correlation between the binding index and any of the conventional spermatological parameters such as motility, morphology and viability of spermatozoa. Although sperm motility is a well-accepted criterion for ejaculate quality, the absence of correlation between motility
and sperm oviduct binding capacity in the present study is not surprising. *In vivo*, motility is important for overcoming the barriers in the female reproductive tract (HUNTER, 1995; SUAREZ, 1996). However, sperm motility apparently has no crucial importance for the quantitative success of sperm–oviduct binding *in vitro*, possibly due to the free accessibility of explants. Furthermore in the present study the viable-motile- and morphologically normal spermatozoa were selected by passing through discontinuous percoll gradients. Our results are inconsistent with that reported by PETRUNKINA et al. (2001b) who found a negative correlation between binding indexes and both the percentage of morphologically abnormal spermatozoa and percentage of spermatozoa with cytoplasmic droplets in boar semen. Our results also disagreed with that reported by THOMAS et al. (1994b) who demonstrated that spermatozoa of stallion ejaculates with low percentage of pathological alterations bound to a higher extent to cultured epithelial cells than did spermatozoa of ejaculates with a high percentage of such alterations. The species differences and the type of semen preparation technique may account for these discrepancies. In the present study frozen-thawed semen was used. Additionally high portions of dead and morphologically abnormal spermatozoa were eliminated from the semen samples by means of a discontinuous Percoll gradient centrifugation technique while in the previous studies no Percoll-selected spermatozoa were used.

5.2 SPERM CHROMATIN STATUS (mf-SCSA)

Defective sperm DNA may not affect fertilization, but may cause great developmental defects later. Consequently, it is important to employ sensitive procedures to select bulls that produce sperm with high DNA integrity (SHEN et al., 1999; IRVINE et al., 2000; VAN DER SCHANS et al., 2000). The results of the present study revealed a narrower range (3.1 - 7.9 %) and a lower overall mean percentage (4.6 ± 1.4 %) of spermatozoa with unstable chromatin in semen samples than that recorded in many other studies. It has been shown that the extent of bull sperm DNA denaturation in the samples tested in SCSA varied from 5 % to 40 % (BALLACHEY et al., 1987) or even from 3 to 91.4 % (SAILER et al., 1996). Similar wide variations have been found in other
studies on bull semen (EVENSON et al., 1980; BALLACHEY et al., 1988), also BOCHENEK et al. (2001) reported that the proportion of bull spermatozoa with defects in chromatin structure varied from 2.1 % to 23.8 %. Furthermore, EVENSON et al. (1984) found that in human semen, spermatozoa with chromatin that was susceptible to in situ denaturation can account for even 100 % and this is probably associated with the fact that sperm chromatin is more sensitive to denaturation in humans than in other species (LOVE and KENNEY, 1999). This may be due to the fact that bulls in artificial insemination centres are generally selected for fertility, therefore the percentage of spermatozoa with unstable chromatin are lower in bulls than in other species like horse and human.

5.2.1 Relationship between sperm chromatin status (mf-SCSA) and sperm volumetric parameters (m-HOST)

To our knowledge this is the first trial to investigate the direct relationship between the sperm chromatin status estimated by mf-SCSA and the sperm volumetric parameters (functional integrity of sperm membrane) estimated by a computer assisted modified hypo-osmotic swelling test (m-HOST). A significant negative correlation was found between relative volume shift (RVS) and percentage of spermatozoa with unstable chromatin in the ejaculate. This relationship could be discussed on the base of common factors, which may exert effects on both chromatin condensation status and membrane integrity of spermatozoa in a given semen sample, such as 1) the amount of reactive oxygen species (ROS) in the ejaculate, 2) stress exerted on spermatozoa by freeze-thawing procedures and 3) the maturation status of the spermatozoa in a given sample.

The generation of excess amounts of ROS by damaged human spermatozoa and leukocytes has been implicated in the control of normal sperm function (AITKEN et al., 1989a; DE LAMIRANDE and GAGNON, 1993) and in the aetiology of male infertility associated with loss of plasma membrane function, altered motility, reduced sperm–zona pellucida binding and acrosomal exocytosis (JONES et al., 1979; ALVAREZ et al., 1987; AITKEN et al., 1989b, 1992). Additionally, it has been recently reported that ROS (endogenously generated or provided as an exogenous stimulus)
can cause an increase in DNA fragmentation in human spermatozoa (AITKEN et al., 1989a; LOPES et al., 1998; TWIGG et al., 1998). Furthermore, the ROS have been hypothesized to play a causative role in the aetiology of defective spermatozoa function through peroxidation of the unsaturated fatty acids within the sperm plasma membrane (AITKEN et al., 1992). Concerning the effect of freeze-thawing procedures on both sperm membrane integrity and chromatin stability, it has been reported that the cellular damages occurred during freezing are usually attributed to membrane rupture caused by either the formation of intracellular ice crystals during rapid cooling or by mechanical force from extra-cellular ice during slow cooling (MOSSAD et al., 1994). It has previously been reported that freeze–thawing causes significant damage to chromatin, membrane integrity and morphology of spermatozoa in both fertile and infertile men (HAMMADEH et al., 1999, 2000). Other researchers reported that freeze–thawing processes resulted in variations in the compactness of mammalian spermatozoa nuclei and over-condensation of spermatozoal DNA (ROYERE et al., 1988; ANZAR et al., 2002). These changes in both sperm membrane and sperm DNA may account for the decreased conception rates following insemination using frozen–thawed semen or for failure of conception despite good post-thaw sperm motility. It has also been reported that loss of plasma membrane asymmetry and occurrence of DNA fragmentation are early processes, which precede the breakdown of plasma membrane integrity and loss of cell viability during apoptosis (KOOPMAN et al., 1994; MARTIN et al., 1995; WYLLIE, 1980). Moreover the presence of spermatozoa with damaged DNA in the ejaculate may be indicative of the occurrence of apoptosis during spermatogenesis (BILLIG et al., 1996; PENTIKAINEN et al., 1999; SAKKAS et al., 1999).

Concerning the maturation status of spermatozoa and its effect on both chromatin status and membrane integrity, it has been emphasized that not only the nucleus but also the sperm membrane are involved in the sperm maturation process in the epididymis (AMANN et al., 1993; JONES, 1998). Furthermore, it has been reported that the epididymal (immature) spermatozoa are characterized by not condensed-, unstable chromatin (BEDFORD et al., 1973; LÖHMER, 2003).
5.2.2 Relationship between sperm chromatin status and *in vitro* fertility

The ability of sperm to fertilize is not only closely correlated to its morphology (KRUGER *et al.*, 1986) but also to the quality of the chromatin packaging (SAKKAS *et al.*, 1996; BIANCHI *et al.*, 1996; MANICARDI, *et al.*, 1995). Up till now the available information about the possible fertility relevance of bull ejaculates with high proportion of unstable sperm chromatin measured in SCSA relies on the non-return rate (BALLACHEY *et al.* 1987; SAILER *et al.*, 1996; BOCHENEK *et al.*, 2001). In the present experiment we used the IVF system to study the relationship between the sperm chromatin status and fertility potential of five AI bulls because this system offers the possibility to follow up the early *in vitro* embryo development up to the blastocyst stage and postulate the disturbances in the embryo development that may be caused by high percentage of spermatozoa with unstable chromatin. Although the non return rate (NRR) *in vivo* fertility parameter have advantages, it is controversial for the evaluation of the paternal fertility of a given bull, as it may be affected by many factors such as age, parity and health status of the inseminated cows, inseminator, as well as the insemination interval and breeding season. Moreover a very high number of first inseminations will be needed to achieve it (AMANN and HAMMERSTEDT, 2002). In addition, often no identical ejaculates are used for both the *in vitro* semen analysis and the employment of insemination making the relationship between spermatological investigation and the fertility more difficult (AMANN and HAMMERSTEDT, 2002).

To ascertain the importance of the sperm chromatin stability for the fertility rate and early embryo development, two groups of bulls, the first one (3 bulls) with extreme high portion of spermatozoa with unstable chromatin and the second one (2 bulls) with extreme low portion of spermatozoa with unstable chromatin were tested in an IVF system. Sub-optimal sperm number (0.25 million sperm /ml) was used in order to obtain clear differences in the fertilization capacity among bulls (AMANN and HAMMERSTEDT, 2002). Significant differences were obtained in both cleavage (day-3) and blastocyst (day-8) rates between the two tested bull groups, so a
significant relationship between the IVF data and the mf-SCSA values could be established. Our results are consistent with those of other studies (TEJADA et al., 1984; IBRAHIM et al., 1988; BALLACHEY et al., 1987; GORCZYCA et al., 1993; HOSHI et al., 1996; BOCHENEK et al., 2001), in which a significant relationship between increased proportion of spermatozoa with unstable chromatin in the ejaculate and lowered fertility was reported.

Although the fertilization rate (day-1) was not included in our study as in vitro fertility parameters, the chromatin status seemed to induce adverse effect not only on the cleavage and blastocyst rates but also on the fertilization and pregnancy rates. A significant negative association was found between the percentage of sperm with DNA fragmentation and fertilization rate ($p = 0.008$) and embryo cleavage rate ($p = 0.01$) after IVF (SUN et al., 1997), also a significant negative association was found between the percentage of sperm with DNA fragmentation and the ICSI fertilization rate (LOPES et al., 1998).

In contrast to the strong predictive value of the SCSA for negative pregnancy outcome, fertilization rate in human was not found to be associated with sperm DNA fragmentation or high DNA stainability (SAKKAS et al., 1996; TWIGG et al., 1998; MORRIS et al., 2002; LARSON-COOK et al., 2003). This indicates that the chromatin defect spermatozoa can penetrate the zona pellucida and fertilize the ova resulting in loss of early embryos and negative pregnancy outcome (LARSON-COOK et al., 2003).

### 5.3 SPERM VOLUMETRIC PARAMETERS (m-HOST)

The most important mechanisms of fertilization, such as capacitation, acrosome reaction and binding of spermatozoa to the oocytes are believed to depend on the functional integrity of the sperm membrane. Therefore, an appropriate assay set to examine the fertilizing capacity of an ejaculate should include evaluation of the sperm membrane integrity. The assessment of membrane integrity is based on examination of the morphology, motility and hypo-osmotic swelling of spermatozoa (JEYENDRAN et al., 1984), supravital stains and flowcytometric techniques (RODRIGUEZ-MARTINEZ et al., 1997). In the current study significant differences among bulls in
the two volumetric parameters (RVS and RVD) were detected. These results are consistent with previous findings reported in bull by PETRUNKINA et al. (2001c) and in human by PETZOLDT and ENGEL (1994) who mentioned that differences in cell volume distribution among human ejaculates could be related to conventional sperm parameters. In the present study we observed that the sperm volume remain constant (stable) when spermatozoa were incubated in iso-osmotic HBS medium but incubation of spermatozoa in hypo-osmotic HBS medium resulted in a quick swelling of the sperm cells showing what is called relative volume shift (RVS). The sperm volume reached to a maximum value at about 5 min, after that it was decreased slowly in a curvilinear manner until reaching nearly to the original volume under iso-osmotic conditions comprising what is called regulatory volume decrease (RVD). These results are in agreement with the results reported previously in bull, boar and dog semen (KULKARNI et al., 1997; PETRUNKINA et al., 2001c,d; PETRUNKINA et al., 2004).

Although several different ion transport mechanisms may play a role in volume regulation, in most mammalian cell types regulatory volume decrease is mediated primarily via a volume-activated opening of independent $K^+$ and $Cl^-$ channels. KULKARNI et al. (1997), PETRUNKINA et al. (2001d) and YEUNG et al. (1999) extend this deduction to bull, boar and mouse spermatozoa. As a cell swells in a hypo-osmotic environment, the channels are activated to allow these ions to exit the cell, thereby reducing internal osmolality and reversing swelling. Moreover our study revealed a stability of the volumetric data on replicates of frozen-thawed ejaculates of the same bull. The value of the relative volume shift provides a continuous quantitative parameter of the osmotic response of individual bulls whereas the classical HOST concerns only the percentage of a qualitative response (swollen or non-swollen) to the hypo-osmotic conditions (PETRUNKINA et al., 2001d). Moreover, the volume distribution shape was reported to be related to the spermatological parameters of the human ejaculates (ENGEL and PETZOLDT, 1994 PETZOLDT and ENGEL, 1994) and seems to be a crucial characteristic of semen quality both for boar and bull semen as reported by LEIDING (1996).
5.3.1 Relationship between sperm volumetric parameters (m-HOST) and viability of spermatozoa (SYBR14/PI)

The functional and structural integrity of sperm membrane are crucial for the viability of spermatozoa. The commonly used staining test (eosin + nigrosin) for assessing sperm membrane measures only its structural integrity. Recently a double, supravital stain consists of PI and SYBR14 was used. The two stains, SYBR-14 (a green, membrane-permeable stain) and PI (a red, membrane-impermeable counter stain) have the same cellular target, the sperm DNA. The hypo-osmotic swelling test (HOST) enables to evaluate the functional status of the sperm membrane. The principle of HOST is based on water transport across the sperm tail membrane under hypo-osmotic conditions.

The results of the present study revealed no correlation between the sperm volume regulatory parameters RVS and RVD (functional integrity of sperm membrane) and sperm viability assessed by SYBR14/PI (structural integrity of sperm membrane). The lack of direct correspondence between the SYBR14/PI sperm viability assay and sperm volume regulation ability indicate that both are independent assays, but both assays indicate that sperm membrane damage is present and the number of membrane-damaged spermatozoa varied among samples from different bulls. In accordance, in several studies utilizing the HOST a sub-population of viable spermatozoa with non-functional membrane was reported (VAZQUEZ et al., 1997; PEREZ-LLANO et al., 2001; LECHNIAK et al., 2002). Besides HOST assess the integrity of the plasma membrane along the sperm flagellum whereas fluorescent dyes assess the head membrane. In contrast, the correlation between the percentage of swollen sperm tails determined morphologically by the classical HOST and the sperm viability determined by dual staining has been reported in canine (RODRIGUEZ-GIL et al. 1994). In human VAN DER SAFFELE et al. (1992), reported that the capacity of fresh spermatozoa to react in a hypo-osmotic environment provided the same information as the viability test using eosin-nigrosin staining, which evaluates the capacity of the head membrane to exclude dye. Furthermore, the results of the present study revealed no significant correlations between sperm volumetric parameters (RVS and RVD) and standard spermatological parameters.
(motility and morphology). In contrast to our results, it has been reported that sperm volume distribution shape is related to classical spermatological parameters of human ejaculates (VAN DER SAFFELE et al., 1992; ENGEL and PETZOLDT 1994; PETZOLDT and ENGEL 1994). These discrepancies may be due to species differences and/or experimental conditions.

In the present study, Percoll selected spermatozoa were used and it was established that when semen sample passed through 2 layers of discontinuous Percoll gradients, large portions of non-motile, morphologically altered and dead spermatozoa could be eliminated (PARRISH et al., 1995).

5.3.2 Relationship between volumetric parameters (m-HOST) and fertility (IVF)

Adequate numbers of progressively motile spermatozoa with biochemically active membranes are required for successful fertilization (JEYENDRAN et al., 1984). In the present study no correlation could be found between in vitro fertility parameters (cleavage and blastocyst rates) and sperm volume regulatory parameters (RVS and RVD). The results of the present study are consistent with that reported by CHAN et al. (1988, 1989), BILJAN et al. (1996) and ROTA et al. (2000) who have found no correlation between normal or abnormal classical HOST results and the success of in vitro fertilization. On the contrary, our results are inconsistent with that reported in bull by PETRUNKINA et al. (2001c) who used the NRR as fertility parameter and in human by (CHECK et al., 1995; VED et al., 1997; ZEYNELOGLU et al., 2000; TARTAGNI et al., 2002).

VAN DER VEN et al., (1986) found a close correlation between the percentage of swollen human spermatozoa and the percentage of denuded hamster oocytes that were penetrated by capacitated spermatozoa. In bovine the suitability of the HOST as a predictive tool for in vitro fertility has not yet been established. Furthermore the HOST evaluates the membrane function, but other variables may be implicated in the fertility potential, especially under in vitro conditions. This may explain the absence of a significant correlation between m-HOST parameters and in vitro fertility parameters in the present study. The type of fertilization procedure, data interpretation, and
statistical analysis may account for these discrepancies. Recently, it has been hypothesized that a defect in the functional integrity of the sperm membrane, which is detectable by the HOST, may reduce fertility potential by causing implantation disorders rather than fertilization problems (CHECK et al., 2001). Subtle abnormalities in sperm detected with the HOST may lead to subsequent abnormal membrane function in the embryo and anomalies in the cell-to-cell communication and binding that seem to have an important role in the attachment of the blastocyst and subsequent penetration of the surface epithelium of the endometrium (DENKER, 1993).

5.4 CONCLUSION

The individual bull difference in their capacity to bind to oviductal epithelium indicates a selective function of sperm-oviduct binding. It is also suggested that an increased percentage of spermatozoa with unstable chromatin coincides with a disturbed plasma membrane functions as indicated by altered sperm volume regulation and reduced BI. Moreover the determination of the capacity of sperm to bind to oviductal epithelium could become a reliable in vitro method for predicting the fertility of a given sire. Furthermore, it can also be concluded that the modified fluorescence microscopical sperm chromatin structure assay is a reliable test to detect sperm chromatin stability as an independent fertility relevant parameter in the bull.
6 SUMMARY

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BINDING CAPACITY OF BULL SPERMATOZOA TO OVIDUCTAL EPITHELIUM IN VITRO AND ITS RELATION TO SPERM CHROMATIN STABILITY, SPERM VOLUME REGULATION AND FERTILITY

The present study was undertaken to evaluate the efficiency of the sperm-oviduct explant binding assay (OEA) in predicting the sperm fertilization potential in vitro and, to investigate the relationship between the sperm oviduct binding ability, sperm response to hypo-osmotic stress and sperm chromatin stability. 90 ejaculates from 30 test bulls were submitted to: the Oviductal-Explant-Assay (OEA), modified fluorescence microscopical Sperm-Chromatin Structure Assay (mf-SCSA), modified Hypo-Osmotic Swelling Test (m-HOST) and viability assessment using SYB14/Probidium Iodide double staining (Sperm Viability Kits). As well as were submitted to morphological and motility evaluation using Cell Motion Analyser (CMA). The number of spermatozoa that bound to 0.01 mm$^2$ of oviductal explant (Binding index; BI) was used as the parameter of binding capacity. Significant (p<0.05) differences in BI and sperm volumetric parameters among bulls were reported in the present study. The BI was positively significant correlated with all sperm volumetric parameters (RVS and RVD) that recovered from the m-HOST and negatively significant (p<0.05) correlated with percentage of spermatozoa with unstable chromatin. No significant correlations were found between the BI and most of the conventional spermatological parameters. The percentage of spermatozoa with unstable chromatin was significantly (P<0.05) negative correlated with relative volume increase, but not correlated with regulative volume decrease (RVD). No significant correlations were recorded between most of the conventional spermatological parameters and both sperm volumetric parameters and percentage
of spermatozoa with unstable chromatin, except for forward motility % of Percoll selected spermatozoa, where a significant (p<0.05) negative correlation was recorded. Six ejaculates of bulls with low (n = 3) and high (n = 3) binding index and five ejaculates of bulls with low (n = 2) and high (n = 3) percentage of spermatozoa with unstable chromatin were further examined for their fertility by the in vitro fertilization (IVF) test. Concerning the relation between the BI and fertility in vitro, no significant difference in cleavage rate was recorded between the two groups of bulls, while a significant (p<0.01) difference in blastocyst rate was recorded, as the bulls with high BI (26.6±5.8) showed high blastocyst rate (16.6±5.8) and the bulls with low BI (8.3±1.7) showed low blastocyst rate (10.9±1.3).

Regarding the relationship between chromatin stability and in vitro fertility, significant differences in both cleavage rate (P<0.05) and blastocyst rate (P<0.01) were recorded between the two groups of bulls, where the bulls with low percentages (2.8 ±0.5) of sperm with unstable chromatin showed significantly higher cleavage- and blastocyst rate (62.5±0.5, 26.4±5.7 respectively) than bulls with high percentages of sperm with unstable chromatin (9±0.8) that showed cleavage rate of 50.7±9.1 and blastocyst rate of 16.1±7.8.

In conclusion, the individual bull difference in the BI indicates a selective function of sperm oviduct binding. It is also suggested that an increased percentage of spermatozoa with unstable chromatin coincides with a disturbed plasma membrane function as indicated by altered sperm volume regulation and reduced BI. Moreover determination of the capacity of sperm to bind oviductal epithelium could become a reliable in vitro method for predicting the fertility of a given sire.
ZUSAMMENFASSUNG

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BINDUNGSFÄHIGKEIT VON BULLENSPERMIEN AN OVIDUKTEPITHEL IN VITRO UND DEREN BEZIEHUNG ZUR SPERMIENCHROMATINSTABILITÄT, VOLUMENREGULIERUNG UND FRUCHTBARKEIT IN VITRO

Ziel der vorliegenden Studie war, eine mögliche bullenspezifische Bindungsfähigkeit von Spermien an Oviduktepithel in vitro zu untersuchen und das Verhältnis zwischen der Bindungsfähigkeit der Samenzellen und der Fähigkeit zur Volumenregulation unter hypoosmotischen Bedingungen sowie zur Stabilität des Spermienchromatins zu ermitteln.

Tiefgefrorenes Sperma von 90 Ejakulaten von 30 Testbullen wurden hinsichtlich der Bindungsfähigkeit im Ovidukt Explant Assay (OEA), der Widerstandsfähigkeit der Spermien DNA gegenüber saurer Denaturierung in situ mit dem modifizierten fluoreszenzmikroskopischen Spermien-Chromatin-Struktur-Assay (mf-SCSA) und der volumenregulatorischen Spermienmembranfunktion mit dem modifizierten Hypo-Osmotischen Schwell-Test (m-HOST) untersucht. Zusätzlich wurde die Spermienmorphologie, die computergestützte Motilität sowie die Permeabilität der Plasmamembran für die Farbstoffe SYBR14/Propidium Jodid (Sperm viability Kits) ermittelt. Mit Sperma ausgewählter Bullen wurde ein in vitro-Fertilisationstest durchgeführt.

Es gab eine signifikante (p<0,05) positive Korrelation zwischen dem Bindungsindex und volumetrischen Parametern (RVS und RVD). Zwischen dem Bindungsindex und dem Prozentsatz an Spermien mit instabilem Chromatin bestand eine signifikante (p<0,05) negative Korrelation. Es gab eine signifikante (P<0,05) negative Korrelation zwischen den Prozentsatz von Spermien mit instabilem Chromatin und der relativen Volumenverschiebung (RVS). Dahingegen konnte keine signifikante Korrelation
zwischen der Chromatininstabilität und der regulativen Volumenabnahme (RVD)
festgestellt werden
Bindungsindex, Volumenregulationsparameter sowie Chromatininstabilität zeigten
keine Korrelation zu den standardspermatologischen Parametern.
Sechs Ejakulate von sechs Bullen mit niedrigem (n = 3) und hohem (n = 3)
Bindungsindex (BI) und fünf Ejakulate von fünf Bullen mit niedrigem (n = 2) und
hohem (n = 3) Prozentsatz an Spermien mit instabilem Chromatin wurden hinsichtlich
ihrer Fertilität im in vitro Befruchtung Test (IVF) überprüft. Bullen mit hohem
Bindungsindex (26.6±5.8) wiesen signifikant (p<0,01) höhere Blastozystenraten auf,
als Bullen mit niedrigem BI (8.3±1.7); zwischen den Teilungsarten bestand kein
signifikanter Unterschied. Bullen mit niedrigen Prozentsätzen chromatininstabiler
Spermien (2.8±0.5 %) zeigten signifikant (p<0,01) höhere Teilungs- und
Blastozystenraten als Bullen mit erhöhtem Vorkommen chromatininstabiler Spermien
(9±0.8 %).
Folgende Schlussfolgerungen werden gezogen: Mit dem Ovidukt Explant Assay steht
möglicherweise ein Testsystem zu Verfügung, welches bullenspezifische,
fertilitätsrelevante Eigenschaften an der Plasmamembran von Spermien erfasst, die
durch standardspermatologische Parameter nicht diagnostizierbar sind. Ein erhöhter
Prozentsatz von Spermien mit instabilem Chromatin geht mit einer gestörten
Plasmamembranfunktion einher, wie durch die veränderte
Spermienvolumenregulation im hypoosmotischen Schweltest und verringerten
Bindungsindex im Ovidukt Explant Assay angezeigt wird.
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9 APPENDIX

9.1 LABORATORY NEEDS

- Dist. Water Preparation system, (Umkehr-Osmose-Anlage)
- Slides (76 x 26 mm)
- Cover slides (50 x 24 mm) and (18 x 18 mm)
- Disposable filter, 0,2 µm (Minisart®)
- Eppendorf-Reaction tubes, 1,5 ml
- Fluorescence microscope with phase Contrast equipment
- Glass centrifuge tubes with conical bottom
- Refrigerator
- Laboratory bottles with thread, 250, 1000 ml
- Laboratory balance, Typ L310
- Laboratory centrifuge (Megafuge 2.0 R)
- Parafilm®
- Phase contrast microscope with warm stage
- Phase contrast microscope without warm stage
- Pipettes, adjustable (automatic pipettes) (100-1000 µl, 20-200 µl, 1-20 µl)
- Tips of pipettes (blue and yellow)
- Warm plate (Hotplate SH 2 D)
- Water bath with temperature control
- Water-operated vacuum pump (Haake)
- Counting chamber

- Co. Kloos, Langenhagen, HANNOVER
- Community of interests of the laboratory specialist trader GmbH & Co KG (IDL), Nidderau
- Co. Sartorius GmbH, Göttingen
- Co. Greiner, Frickenhausen
- Co. Zeiss (Axioskop), Jena
- Co. Sarstedt, Nümbrecht
- Co. Liebherr, Nürnberg
- Co. Jürgens (9.072 0025), (9.072 010), HANNOVER
- Co. Sartorius GmbH, Göttingen
- Co. Heraeus Instruments, Hamburg
- Co. American National Can™, USA
- Co. Zeiss (1697), Jena
- Co. Zeiss, Jena
- Co. Abimed, Langenfeld
- Co Greiner (685290und686290), Frickenhausen
- Co Stuart Scientific, England
- Co for laboratories GmbH (GFL), Burgwedel
- Co Landgraf Laborgeräte, Langenhagen
- Thoma Co. Glass wares Hecht, Sontheim
9.2 CHEMICALS AND REAGENTS

- BSA (Cohn’s Fraktion V)
- Calciumchloride (CaCl₂)
- EDTA
- Gentamycinsulfate
- Glucose
- Hepes
- Kaliumchloride (KCl)
- Kaliumhydroxide (KOH)
- Kaliumdihydrogenphosphate (KH₂PO₄)
- Magnesiumchloride (MgCl₂)
- Natriumbikarbonate (NaHCO₃)
- Natriumchloride (NaCl)
- Natriumcitrate
- Natriumlactate
- Natriumdihydrogenphosphat (NaH₂PO₄)
- Dinatriumhydrogenphosphat (Na₂HPO₄)
- Natriumpyruvate
- Percoll®
- Propidiumiodide (PI) & SYBR14
- Streptomycine (Strepto-Hefa)
- tri-Natrium citrate
- Ethanol (96%)

- Co. Merck (1.12018.0025), Darmstadt
- Co. Merck (1.02382.0500), Darmstadt
- Co. Merck (1.08418.0100), Darmstadt
- Co. Serva (22185.02), Heidelberg
- Co. Merck (1.04074.0500), Darmstadt
- Co. Merck (1.10110.0250), Darmstadt
- Co. Merck (1.04938.0500), Darmstadt
- Co. Merck (1.05033.0500), Darmstadt
- Co. Merck (1.04873.0250), Darmstadt
- Co. Merck (1.05833.1000), Darmstadt
- Co. Merck (1.06329.0500), Darmstadt
- Co. Merck (1.06448.0500), Darmstadt
- Co. Fluka AG, Schweiz
- Co. Merck (1.06346.0500), Darmstadt
- Co. Merck (1.06586.0500), Darmstadt
- Co. Merck (1.06619.0500), Darmstadt
- Co. Sigma-Aldrich (P-1644),Deisenhofen
- Co. Hefa Pharm GmbH & Co KG, Werne
- Co. Merck (1.06448.1000), Darmstadt
- Co. CG Chemicals, Laatzen
9.3 BUFFERS, SOLUTIONS, MEDIA AND DILUENTS

9.3.1 Natrium chloride solution 10 % (for killing of spermatozoa)

- 10 gm NaCl /100 ml D.W. (to kill spermatozoa for estimation of sperm cell density

9.3.2 Formolcitrat (for fixation of spermatozoa)

4 parts of 37 % formaldehyde solutions are mixed with 96 parts of a 2.9 % Natrium citrate solution. This solution is suited to the liquid (wet) fixation of spermatozoa.

9.3.3 Heps buffer saline (HBS) for m-HOST

a) 300 mOsmol (isotonic solution):

- NaCl 137.0 mM (8.0 g / L)
- Glucose 10.0 mM (1.8 g / L)
- Heps 20 mM (4.77 g / L)
- KOH 2.5 mM (2.5 ml 1M / L)

b) 180 mOsmol (hypotonic solution):

- 600 ml HBS isotonic (300 mOsmol) + 400 ml dist. water (0 mOsmol)

- Both iso- and hypo-tonic solutions were sterile filtered before use.

9.3.4 Percoll® Solution (after HARRISON et al., 1993)

1. 10-times concentrated stock solution of Hepes Saline

- NaCl 137 mM (8.0 g /100ml)
- Heps 20 mM (4.77 g/100 ml)
- Glucose x H2O 10 mM (1.8 g /100ml)
- KOH (1 M) 2.5 ml/100ml

- All above chemicals dissolved in 70 ml dist. Water
- The pH was adjusted to 7.4
- Add dist water to 100 ml and adjust the pH to 7.6
-This solution divided into 10.8 g in plastic tubes and frozen stored.

2. **Solution M**

10.8 g of solution I (Hepes saline stock solution) was filtered with sterile filter and mixed with dist water to reach a volume of 100 ml. 250 µl gentamycine sulphate (conc. 20mg / ml) was added. The pH adjusted to 7.4 and the Osmolarity was measured, measuring value → m (should around 295 mOsm/kg)

3. **Percoll®-undiluted**

Percoll was taken from the refrigerator and the osmolarity was measured, measuring value → p (should around 15 mOsm / kg)

4. **Solution 1 + 9**

-5.4 g of the solution1 (Hepes saline stock solution) was filtered through in sterile filter and mixed with 50.9 g from undiluted Percoll®.

-The osmolarity was measured; measuring; value → dp (350mOsm/kg)

5. **Computation**

1.) \( O_a = 0,1 \times (10m + 9p) = \sim 310 \)

2.) \( R = \frac{[Qa - (0,1 \times dp)]}{(0,9 \times dp)} = \sim 0,85 \)

3.) \( V_p = \frac{(10m - 295)}{[R \times (295 - p)]} = \sim 11 \)

6. **Percoll®. -Working solutions**

a) 100 %age Percoll® - working solution:

= \( (Vp - 9) \times 1.13 \times 5 \) g Percoll® + solution 1 + 9

= \( (Vp - 9) \times 5.65 \) g Percoll® + solution 1 + 9

-Carefully shacked

- The osmolarity should around 295 mOsm/kg
b) 45 %age Percoll® - working solution:
- One half (ca. 33 ml) of the 100 % -solution (V₁ corresponds) was taken and
  with solution M filled to V₂
  \[ V₂ = V₁ \times \frac{100}{45} \]  (Carefully shaken)

c) 90 %age Percoll® - used solution:
- Another half (ca. 33 ml) of the 100 % -solution (V₁ corresponds) was taken
  and with solution M filled to V₃
  \[ V₃ = V₁ \times \frac{100}{90} \]
  - Carefully shaken
  - The prepared solutions can be stored at 4°C in the refrigerator and used for
    approximately two weeks

9.3.5 Hepes. 0.1 % BSA-buffer for Supra vital staining

  - 0.76 g NaCl
  - 0.03 g KCl
  - 0.252 g Fructose
  - 0.15 g CaCl₂
  - 0.01g MgCl₂
  - 100 ml Dist. water
  - This buffer portioned into 10 ml aliquots and stored at 23°C

9.3.6 Materials for mf-SCSA

9.3.6.1 Natrium citrate buffer 2.9 % (1000 ml)

  - 29 g Tri-Natrium-citrat-dihydrat
  - 3.36 g EDTA
  - 1000 ml dist. water
  - The buffer stored at the room temp and can be used up to 3-4 weeks

9.3.6.2 Carnoy’s solution, pH 2 (60 ml)

  - 20 ml acetic acid DAB mixed in 40 ml Methanol 99.9 %.
9.3.6.3 Citric acid-stock solution 0.1 molar (1000 ml)
- 21.01 g citric acid -monohydrate DAB
- 1000 ml dist. water. Stored in the refrigerator and can be used up to 3-4 weeks

9.3.6.4 Di-Natriumhydrogenphosphat, 0.3 molar solution (100 ml)
- 5.34 g Na2HPO4 x H2O
- 100 ml Dist. water.
- The buffer stored in the refrigerator and can be used up to 3-4 weeks

9.3.6.5 Acridin Orange (AO) stock solution (100 ml)
- 0.1 g Acridin Orange (pure substance)
- 100 ml dist. water.
- The solution stored dark bottle in the refrigerator and can be used up to 3-4 week
9.4 MATERIALS FOR THE OVIDUKT-EXPLANT-ASSAY

9.4.1 Equipments and instruments

- Anatomical tweezers
- Artery forceps
- CO2 incubator (CO2-Auto-Zero)
- Computer connected with
- Monitor (Triniton)
- Computer assisted surface area estimation programme „Aida“
- One used disposable insulin syringes (1 ml)
- Inverted microscope (IM 35) coupled with
- Video camera (Kappa, CF 8 / 1)
- Monitor (WV-3M 1400)
- Petri dishes, size (35 x 10 & 100 x 15 mm)
- Scissors with two-pointed thighs
- Silicon (Silicone Stopcock Grease, Dow Corning)
- Scale (OT, 0,01 mm)
- Stereomicroscope
- Videocassettes (300 min)
- Video recorder (SLV-E 720, VHS)
- Warm plate (HT 200)

-Co. TBH GmbH, Langenhagen, Hannover
-Co. Aesculap, TBH GmbH, Langenhagen, Hannover
-Co. Heraeus, Hamburg
-Co. Dell, USA
-Co. SONY, JAPAN
-Co. Mika medica GmbH BildanalyseVers. 2.0, Copyright 1992), Rosenheim
-Co. Heiland, Hamburg
-Co. Zeiss, Jena
-Co. Panasonic, JAPAN
-Co. SONY, JAPAN
-Co. Greiner GmbH, Frickenhausen
-Co. Aesculap, TBH GmbH, Langenhagen, Hannover
-Co. Serva Feinbiochemica, Heidelberg
-Co. Olympus, JAPAN
-Co. Zeiss (475003), Jena
-Co. SONY, JAPAN
-Co. SONY, JAPAN
-Co. Minitüb GmbH, Heidelberg
9.4.2 PBS Medium (LEFEBVRE and SUAREZ, 1996)

- 0.7948 g NaCl
- 0.0199 g KCl
- 0.0047 g MgCl$_2$
- 0.1321 g NaH$_2$PO$_4$
- 0.02 g KH$_2$PO$_4$
- 0.0097 g CaCl$_2$
- 100 ml dist. water.

The saline solution could up to 3 months in refrigerator used after adjust the pH to 7.4 and the osmolarity (~290 mOsmol / kg)

9.4.3 TALP Medium (PARRISH et al. 1988)

- 0.5844 g NaC
- 0.0231 g KCl
- 0.21 g NaHCO$_3$
- 0.0041 g NaH$_2$PO$_4$
- 0.0222 g CaCl$_2$
- 0.0082 g MgCl$_2$
- 0.242 g Natrium lactate
- 0.11 g Natrium pyruvate (freshly added at the day of experiment)
- 0.2385 g Hepes (freshly added at the day of experiment)
- 0.6 g BSA (Cohn.s Fraktion V, freshly added at the day of experiment)
- 0.005 g Gentamycin (freshly added at the day of experiment)

- The pH of the prepared solution should be adjusted to 7.54 and the osmolarity should be between (~280-290 mOsm / kg).
- At the day of the experiment, the working medium should be at least incubated for one hour in the CO$_2$ incubator (39 °C and 5 % CO$_2$)
9.5 MATERIALS FOR IVF

9.5.1 PBS stock solution (1000 ml)
- 3.6 g Natrium pyruvate
- 5.0 g Streptomycin sulphate
- 100.0 g Glucose
- 13.3 g CaCl$_2$ 2H$_2$O
- 58.8 g Penicillin (Sodium)
- 1000 ml dist. water

9.5.2 Slice medium (500 ml)
- 500 ml PBS
- 0.5 g BSA
- 0.0056 g Heparin

9.5.3 TCM-air-Medium (100 ml)
- 1.510 g TCM 199
- 0.005 g Gentamycin
- 0.035 g NaHCO$_3$ (extra solved)
- 0.0022 g Natrium pyruvate
- 0.100 g BSA (added after adjustment of the pH to 7.2)
- 100 ml dist. water
- Sterile filtration
The medium stored in the refrigerator and can be used up to 2 weeks

9.5.4 TCM-pure Medium + BSA (Wash drops)
- 1.1510 g TCM 199
- 0.005 g Gentamycin
- 0.220 g NaHCO$_3$ (extra solved)
- 0.0022 g Natrium pyruvate
- 100 ml dist. water
- Stirred in open glass for about one hour till the pH reached to 7.4
- Sterile filtration. Stored in refrigerator and can be used up to one week
- 0.1 g BSA (freshly added)

**9.5.5 TCM-pure Medium+BSA+Suigonan® (Maturation drops)**

- 975 µl TCMpur + BSA
- 25 µl Aliquot Suigonan® in 0.9 % NaCl solved (entspricht 10 IU PMSG and 5 IU HCG). Suigonan® (contains 400 IU PMSG and 200 IU HCG, the dry substance dissolved in 1 ml 0.9 % NaCl and in divided into 25 µl aliquots then stored at -20°C for about one month can be used)

**9.5.6 sperm-TALP stock solution (500 ml)**

- 2.920 g NaCl
- 0.1155 g KCl
- 1.050 g NaHCO₃ (extra solved)
- 0.0205 g Na₂HPO₄·H₂O
- 0.147 g CaCl₂
- 0.019 g MgCl₂
- 0.025 g Gentamycin
- 0.005 g Phenol red
- 2.0175 g Natrium lactate (60 %)
- 1.192 g Hepes (MG 238,3)
- 500 ml Dist. water.
- The pH adjusted to 7.4, sterile filtered and stored in the refrigerator for up to 3 months

**9.5.7 fert-TALP stock solution (500 ml)**

- 3.329 g NaCl
- 0.1195 g KCl
- 1.050 g NaHCO₃ (extra solved)  
- 0.0205 g Na₂HPO₄·H₂O  
- 0.147 g CaCl₂  
- 0.024 g MgCl₂  
- 0.0015 g Penicillamine  
- 0.005 g Phenol red  
- 0.930 g Natrium lactate (69 %)  
- 500 ml dist. water  

The solution ist im Refrigerator drei Monate haltbar

9.5.8 HHE Stock solution (Heparin / Hypotaurin / Epinephrine)

9.5.8.1 250 µM Epinephrine (50 ml)

- 0.165 g Natrium lactate (60 %)  
- Na-metabisulphate (Na₂S₂O₅)  
- 50 ml dist. Water  
- The pH adjusted to 4.0  
- 0.0018 g Epinephrine added to 40 ml of the above solution

9.5.8.2 Hypotaurin 1 mM (10 ml)

- 0.0011 g Hypotaurin  
- 10 ml Dist. water

9.5.8.3 Heparin 50 IE (10 ml)

- 0.0028 g Heparin, Serva 177000 IE or  
- 0.0027 g Heparin, Serva 186000 IE  
- 10 ml dist. water  
- Sterile filtration

9.5.8.4 Working medium-Stock solution (40 ml)

- 4 ml Epinephrine solution  
- 10 ml Hypotaurin solution
- 26 ml dist. water
- 40 µl of the Heparin solution mixed with 80 µl of this Stock solution and divided in Eppendorf cups and stored at –20°C
- The working medium can be used for 3 months

9.5.9 SOF Medium

- 10 ml SOF/Stock A-Medium
- 0.08 g BSA
- 1 Aliquot SOF / Stock B-Medium
- 1 Aliquot SOF / Stock C-Medium
- 1 Aliquot SOF / Stock D-Medium
- 1 Aliquot SOF / Stock E-Medium
- 1 Aliquot SOF / Stock F-Medium
- All mixed together and the pH adjusted to 7.4 and the osmolarity should be around 270 then sterile filtered

9.5.9.1 SOF / Stock A-Medium (100 ml)

- 0.630 g NaCl
- 0.0537 g KCl
- 0.210 g NaHCO₃ (extra solved)
- 0.0163 g KH₂PO₄
- 0.025 g CaCl₂ 2H₂O (extra solved)
- 0.0048 g MgCl₂
- 0.062 g Natrium lactate (60 %)
- 0.027 g Glucose
- 0.001 g Phenol red
- 100 ml Dist. water
- Sterile filtered and stored at 4°C for up to one month
9.5.9.2 SOF / Stock B-Medium (1 ml)
- 0,010 g Natrium pyruvate
- 1 ml dist. water
- The medium portioned into 36 µl aliquots

9.5.9.3 SOF / Stock C-Medium (1 ml)
- 0,015 g Glutamine
- 1 ml dist. water
- The medium portioned into 100 µl aliquots

9.5.9.4 SOF / Stock D-Medium (1 ml)
- 0,010 g Gentamycine
- 1 ml Dist. water
- The Medium is being portioned into 50 µl aliquots in Eppendorf cups
- Stock B, C, and D stored deep frozen at –20°C
- The solution is one month usable

9.5.9.5 SOF / Stock E-Medium (100 ml)
- 1 ml Non-essential aa
- 100 ml Dist. water
- The medium portioned into 100 µl aliquots and stored at 4°C and can be used for up to 3 months

9.5.9.6 SOF / Stock F-Medium (100 ml)
- 2 ml Essential aa
- 100 ml dist. water
- The medium was portioned into 200 µl aliquots and stored at 4 °C and can be used for up to 3 months.
10 STATUTORY DECLARATION

Hereby I explain that I Independently wrote the thesis with the title:

BINDING CAPACITY OF BULL SPERMATOZOA TO OVIDUCTAL EPITHELium IN VITRO AND ITS RELATION TO SPERM CHROMATIN STABILITY, SPERM VOLUME REGULATION AND FERTILITY

During the preparation the following assistance were taken up:
1. The statistic evaluation of the results made after consultation with and under guidance of Ms. Dipl. Dipl.-Phys. Dr. A. M. Petrunkina under use of the statistical program SAS.
2. At Institute for Reproduction Medicine of the Veterinary University Hannover I accomplished the attempts with the assistance and aids specified in the chapter "Materials and Methods`
3. The technical consultation was undertaken from Ms Univ. Prof. Dr. rer. nat. Dr. med. habil. Edda Töpfer-Petersen and Ms. Apl. Prof. Dr. med. vet. Dagmar Waberski -Institute for Reproduction Medicine of the Veterinary University Hannover.
I made the thesis at the following institutes: 1) Institute for Reproductive Medicine of the Veterinary University Hannover and 2) Institute for Animal Science, Mariensee, Federal Agriculture Research Center (FAL).
The thesis was so far not submitted for an examination or a graduation or for a similar purpose for evaluation.
I insure that I gave the managing data accordingly after best knowledge completely and to the truth.
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