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Research

# Kinetic characterization of the changes in protein tyrosine phosphorylation of membranes, cytosolic $Ca^{2+}$ concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells

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**On reaching the oviduct, spermatozoa are retained in the isthmic region of the oviduct until ovulation occurs. The essential steps of capacitation are co-ordinated in this region. In this study, a primary cell culture system of oviductal epithelial cells was established to investigate sperm binding to oviductal epithelium and modulation of sperm function during incubation under capacitating conditions in co-culture with oviductal epithelial cells. Epithelial cells were stripped from the oviducts of sows and cultivated for 5–7 days on Lab-Tek Chamber slides on Matrigel. The preparations on chamber slides and suspensions of control spermatozoa were incubated for 3 h in Tyrode's albumin lactate pyruvate (TALP) medium. At 3, 30, 60, 90 and 180 min the free-swimming spermatozoa were collected by washing, and membrane integrity, tyrosine phosphorylation patterns and $[Ca^{2+}]_i$ of bound, unbound and control spermatozoa were assessed with fluorescent probes (propidium iodide, Cy-3 and fluo-3-AM). The cells bound to oviductal epithelial cells showed reduced cytosolic $Ca^{2+}$ concentration, reduced and almost absent tyrosine phosphorylation of membrane proteins and higher viability at the time of the first sampling. Increases in $Ca^{2+}$ concentration and cell death occurred much more slowly during incubation in cells bound to oviductal epithelial cells compared with free-swimming spermatozoa, and no changes in tyrosine phosphorylation were observed. The preferential binding of viable, low-$Ca^{2+}$ cells with suppressed tyrosine phosphorylation and slower functional modulation of boar spermatozoa attached to oviductal epithelial cells might represent a mechanism for selecting functionally competent spermatozoa and prolonging their lifespan by delaying capacitation in the oviductal reservoir.**

## Introduction

The fertilization of an egg by a spermatozoon is the fundamental event in life, as it is a prerequisite for the creation of a new individual. It includes a highly co-ordinated sequence of cellular interactions between both haploid gametes to ensure the formation of the diploid zygote and the commencement of the developmental programme (Yanagimachi, 1994). In mammals, fertilization takes place in the female reproductive tract. Thus, male and female intercommunication in mammals not only occurs between spermatozoa and egg but also between spermatozoa and the somatic cells encountered during their journey through the female genital tract. Although the cervical barrier, uterus and uterotubal junction have important functions for sperm transport (Overstreet and Cooper, 1978a,b) and for selection of putative functional spermatozoa (Larsson, 1988; Katz *et al.*, 1989; Scott, 2000), it appears that the isthmic region of the oviduct has a special responsibility for spermatozoa. Spermatozoa that have passed through the uterus into the oviduct are retained in the caudal isthmus by binding to the ciliated epithelium lining the duct. Near the time when the egg is ovulated into the ampulla, spermatozoa start to develop a hyperactivated motility, which aids in the release from the oviductal epithelium, and they swim to the site of fertilization (Hunter, 1995; Suarez, 1999). Although fertilization in mammals can also be completed successfully under extracorporal conditions (IVF), the interaction between spermatozoa and the somatic cells of the oviduct has a synchronizing and co-ordinating effect to ensure the meeting of functionally competent gametes at the site of fertilization at the right time. Suarez (1998) proposed that the oviductal sperm reservoir may have a number of functions: firstly, maintenance of the sperm fertilizing competence between oestrus and ovulation; secondly, modulation of capacitation to synchronize sperm

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function with the time of ovulation; and thirdly, control of sperm transport to the ampulla, thereby contributing to the prevention of polyspermic fertilization (Hunter, 1981, 1984; Smith, 1998; Suarez, 1998). For fertilizing competence to be acheived, spermatozoa must reside in the female genital tract for a finite period of time, thereby entering an activated state. The sequence of events accompanying sperm activation has been collectively called 'capacitation' (Austin, 1951; Chang, 1951). Capacitation defines the functional changes that spermatozoa must undergo to respond to the oocyte zona pellucida with the acrosome reaction, thereby enabling them to pass through the zona pellucida and finally fuse with the egg (Yanagimachi, 1994). The current still fragmentary knowledge on the mechanisms underlying capacitation-related changes is derived from studies *in vitro* in defined media. A prerequisite is the removal of the seminal plasma and the coating substances that occupy the sensitive acrosomal region, thereby rendering the newly exposed sperm surface accessible to lipid-binding components such as serum albumin (Visconti *et al.*, 1999). The lipid acceptors extract cholesterol from the sperm membrane, thereby enhancing membrane fluidity and consequently initiating further alterations such as reorganization of the sperm surface (Töpfer-Petersen *et al.*, 1990), uptake of extracellular $Ca^{2+}$ and tyrosine phosphorylation of a subset of sperm proteins (Visconti *et al.*, 1995a,b; Visconti and Kopf, 1998), which is a capacitation-induced event in boar spermatozoa (Flesch *et al.*, 1999). Massive $Ca^{2+}$ influx has been predominantly associated with the exocytotic event of the acrosome reaction. However, a moderate increase in intracellular $Ca^{2+}$ concentrations appears to be necessary to develop capacitation fully (Fraser, 1995). Intracellular $Ca^{2+}$ concentrations increase with hyperactivation, an event associated with capacitation (Suarez *et al.*, 1993; Suarez, 1996) and may be involved in many enzyme-mediated signalling pathways underlying capacitation (Harrison, 1996). It should be noted that increase in $[Ca^{2+}]_i$ and progressing cell death are not independent events. The downregulation of intracellular calcium may serve to prolong the viability of spermatozoa, because capacitation, which involves an increase in $[Ca^{2+}]_i$, can be seen as an ongoing destabilization process leading to cell death if fertilization does not take place *in vivo* and *in vitro* (Hunter, 1987; Harrison, 1996).

In pigs, bicarbonate/$CO_2$ induces early and rapid changes in the lipid architecture in the sperm plasma membrane (Gadella and Harrison, 2000; Harrison and Miller, 2000) that is concomitant with membrane destabilization (Harrison, 1996). These early destabilization events may pave the way for the BSA-mediated cholesterol efflux (Gadella and Harrison, 2000) that, in turn, triggers further changes. Capacitation reduces the lifespan of spermatozoa. According to Harrison (1996), capacitation is a series of positive destabilizing events that allow spermatozoa to undergo the zona-induced acrosome reaction within a limited window of destabilization, or lead to cell death. Thus, the maintenance of viability and the modulation of capacitation by the oviduct, demonstrated in a number of studies using different animal models, are mutually associated events (Smith, 1998). In horses, $[Ca^{2+}]_i$ of spermatozoa attached to oviductal epithelial cells is maintained at lower concentrations than in free-swimming spermatozoa and direct contact with the apical plasma membrane of the epithelial cells seems to be responsible for the maintenance of low $[Ca^{2+}]_i$ (Dobrinski *et al.*, 1996, 1997). These observations indicate that the regulation of $Ca^{2+}$ uptake in spermatozoa is an important feature of oviductal sperm reservoir function. However, Dobrinski and co-workers do not describe the proper kinetics of temporal changes, or characterize the speed of processes and they do not determine the time windows in which the oviduct may slow down or initiate capacitation.

A sperm–oviduct epithelial cell binding assay has been established to enable kinetic characterization of sperm viability and the post-ejaculatory capacitation-related events leading to fertilization in pigs. Fluorescent probes were used to investigate kinetic changes in membrane integrity and, as a feature of ongoing capacitation, changes in $Ca^{2+}$ concentration and tyrosine phosphorylation in spermatozoa co-cultured with oviductal epithelial cells.

## Materials and Methods

### *Materials*

Unless otherwise stated, all chemicals were obtained from Sigma (Deisenhofen) and all materials for cell culture were obtained from Nunc (Wiesbaden).

### *Media*

For the swim-up procedure and sperm dilution, Androhep without EDTA (mod. Androhep) was used (Waberski *et al.*, 1994); it consisted of 144 mmol glucose $l^{-1}$, 27.2 mmol trisodium citrate-2-hydrate $l^{-1}$, 14.3 mmol $NaHCO_3$ $l^{-1}$ and 37.0 mmol Hepes $l^{-1}$ (pH 7.4, 290 mOsm $kg^{-1}$). Coincubations of spermatozoa with the cell cultures and control incubations were performed in a modified Tyrode's balanced salt solution (TALP; Parrish *et al.*, 1988), containing 6 mg BSA $ml^{-1}$ and 2.2 mg sodium pyruvate $ml^{-1}$; before use, it was equilibrated at 39°C for 1 h in a humidified atmosphere containing 5% $CO_2$ (pH 7.4, 300 mOsm $kg^{-1}$). For culture of epithelial cells, 100 ml tissue culture medium (TCM-199) supplemented with 0.0022 g pyruvate, 0.01 g glutamine, 0.005 g gentamycin and 10% fetal bovine serum was used. Spermatozoa loaded with fluo-3-AM were diluted in a Hepes-buffered saline (HBS) containing 137 mmol NaCl $l^{-1}$, 10 mmol glucose $l^{-1}$, 2.5 mmol KOH $l^{-1}$, 20 mmol Hepes $l^{-1}$, 1 mg polyvinylalcohol $ml^{-1}$ and 1 mg polyvinylpyrrolidone $ml^{-1}$ (Harrison *et al.*, 1993) before washing. Sucrose wash medium (SWM) was used as a washing medium, consisting of 200 mmol sucrose $l^{-1}$, 10 mmol NaCl $l^{-1}$, 10 mmol glucose $l^{-1}$, 2.5 mmol KOH $l^{-1}$, 20 mmol Hepes $l^{-1}$, 0.5 mg

polyvinylalcohol ml$^{-1}$ and 0.5 mg polyvinylpyrrolidone ml$^{-1}$ (Harrison *et al.*, 1993). After preparation, all media were passed through a 0.2 μm single use filter unit (Minisart Sartorius, Goettingen).

### *Collection of material and establishment of oviductal epithelial cell cultures in chamber slides*

The oviducts for obtaining oviductal epithelial cells were collected at a local abattoir from sows at different stages of the oestrous cycle. The oviducts were transported in warm PBS (39°C) and stored in the same medium in a 39°C incubator for < 1.5 h.

Under sterile conditions, the mesenteries were removed and the oviducts were divided into 3–4 sections. Two small pairs of tweezers were used to strip the oviductal epithelial cells out of the oviducts by squeezing them from the ampulla to the uterotubal junction. The cells were collected in a droplet of culture medium. For separation, the cells were passed 3–4 times through a syringe with a small needle (0.55 mm × 25.00 mm; Neolus, Leuven). The cells were washed twice in 5 ml TCM-199 by sedimentation for at least 30 min each time.

Four-well chamber slides were covered with Matrigel (Serva, Heidelberg) diluted 1:10 with culture medium. Each well of a chamber slide was filled with 800 μl TCM-199 and 10 μl cell suspension to establish cell cultures. The chamber slides were incubated at 39°C and 5% $CO_2$ in air. The culture medium was changed every 48–72 h.

Cell growth was already observed after 1 day of incubation. After 5–7 days the cultured cells formed nearly confluent monolayers. Only primary cell cultures showing intensive ciliary activity were used for the experiments.

### *Electron microscopy of oviductal epithelial cell cultures*

The oviductal epithelial cells were cultured for 5 days. After this time, the oviductal epithelial cells were fixed by immersion for 24 h in 5% (w/v) glutaraldehyde in sodium cacodylate buffer (pH 7.2). After repeated washing in cacodylate buffer the samples were post-fixed for 1 h in 1% (w/v) $OsO_2$, dehydrated in increasing concentrations of alcohol and embedded in Epon 812. Preliminary orientation and assessment of the findings were carried out by light microscopy of semi-thin sections stained with toluidine blue before ultrastructural examination of ultra-thin sections. The equipment used was a Zeiss EM 10C transmission electron microscope at 60 kV.

### *Animals and semen preparation*

The semen of six fertile boars of the colony of the Institute for Reproductive Medicine was used in this study. The semen (sperm rich fraction) was collected twice a week by the 'gloved hand' method. The gel secretion was removed using sterile gauze. The semen was transferred to the laboratory immediately after collection. The spermatozoa were washed by the swim-up procedure as follows. In each of eight tubes, 1 ml semen was layered underneath 10 ml mod. Androhep. The tubes were incubated for 1 h in a 39°C waterbath. The top 1 ml was removed from each tube and centrifuged for 10 min at 150 ***g***. After centrifugation, the supernatant was discarded and the pellet was re-suspended in mod. Androhep.

For all ejaculates, the conventional sperm examinations (motility, agglutination grade and sperm cell concentration) were made. The percentage of morphologically abnormal spermatozoa was checked to ensure that the quality of ejaculates was within normal ranges. During collection, transfer to laboratory and preliminary manipulations, the temperature was maintained above 25°C to avoid cold-shock. The spermatozoa were used for experiments within 1 h after collection.

### *Coincubation of oviductal epithelial cells and spermatozoa*

For all the experiments described below, washed spermatozoa were coincubated with 5–7-day-old oviductal epithelial cells at 39°C and 5% $CO_2$ in air. Five sets of four-well chamber slides were used in each experiment. The culture medium was replaced in each well by 500 μl TALP medium and $5.0 \times 10^5$ (in about 5 μl mod. Androhep medium) sperm cells were added. As a control, one Eppendorf tube (Eppendorf, Sarstedt) was filled with $5.0 \times 10^6$ sperm cells in 500 μl TALP medium. After 3, 30, 60, 90 and 180 min of coincubation, the bound spermatozoa, unbound spermatozoa and a sample of spermatozoa incubated in TALP in the absence of oviductal epithelial cells (TALP-incubated spermatozoa) were examined.

### *Membrane integrity changes (Expt 1)*

A total of 15 ejaculates from six different boars was used. The membrane integrity of spermatozoa was evaluated using propidium iodide staining (see Harrison and Vickers, 1990). Stock solution (0.5 mg ml$^{-1}$) of propidium iodide (10 μl) was added to the preparations of spermatozoa coincubated with cultured epithelial cells and TALP-incubated sperm cell suspensions and left for 3 min. Afterwards, 500 μl TALP suspension containing unbound spermatozoa was removed from the co-culture preparation. Subsequently, the cultures were washed vigorously with 500 μl TALP twice and the wash medium was added to the medium containing the unbound spermatozoa. The total volume of 1.5 ml TALP (from each of four chambers with coincubated spermatozoa and epithelial cells) was transferred to a centrifuge tube and spermatozoa were collected by centrifugation for 10 min at 150 ***g***. For the TALP-incubated cell suspension, the propidium iodide was added to the suspension to the same final concentration of 10 μg ml$^{-1}$. An aliquot of the TALP-incubated sperm suspension was subjected to the same treatment and the samples were evaluated after both processing procedures to ensure that the collection of unbound spermatozoa by centrifugation does not influence membrane state. The collection of

unbound spermatozoa was performed using the method described for all experiments. For the bound spermatozoa, the frame of the chamber slide was removed, the sample was covered with the coverslip, and membrane integrity was evaluated at each sampling time (3, 30, 60, 90 and 180 min).

Propidium iodide fluorescence was observed using a 546 nm excitation filter.

### *Tyrosine phosphorylation (Expt 2)*

Sperm-rich fractions from five different boars were used. The bound, unbound and TALP-incubated sperm preparations were processed as described above. Air-dried smears of the unbound and control sperm suspensions and slides of co-cultured cells (after frames were removed) were prepared at each sampling time. As an additional control, air-dried slides of native cultures (without added spermatozoa) were prepared. The preparations were fixed in methanol for 10 min. Blocking solution (200 µl; 50% goat serum and 0.1% (v/v) Triton-X100 in PBS) was added to the preparations; the slides were covered with parafilm (American National CanTM, Chicago) and then incubated overnight in a wet chamber (Gelman, Schweden) at 4°C to block non-specific binding. After washing in wash buffer (0.1% (v/v) Triton-X100 in PBS), 15 µl stock solution (50 µg $ml^{-1}$ in antibody buffer consisting of 1% goat serum and 0.1% (v/v) Triton-X100 in PBS) of the primary antibody mouse IgG anti-phosphotyrosine (clone 1G2, Boehringer-Mannheim, Mannheim) was added. The preparations were incubated for 1 h at 37°C in the wet chamber. As a secondary antibody, goat anti-mouse IgG Cy3-conjugate (Jackson ImmunoResearch Laboratories Inc., West Grove) was used. After subsequent washing (3 × 10 min in wash buffer), 80 µl secondary antibody solution (1:400 in antibody buffer) was added, and the preparations were incubated for 1 h at 37°C. After final washing (3 × 10 min in wash buffer) the samples were dried and mounted in mounting medium (PBS:glycerin; 1:9). The Cy3-fluorescence was observed by fluorescence microscopy (excitation filter 546 nm).

Two control series were performed for each of five experiments in this set to check the specificity of binding. In the first series, the preparations were incubated with the secondary antibody only. In the second set of controls, the primary antibody was preincubated with saturated ortho-phosphotyrosine solution for 1 h at 37°C. The anti-tyrosine/ortho-phosphotyrosine solution was added to the slides instead of primary antibody.

### *Changes in cytosolic Ca2+ content (Expt 3)*

Sperm-rich fractions of ejaculated semen from five boars (a total of eight ejaculates), processed and prepared as described above, were used. Changes in cytosolic calcium content were recorded using the fluorescent probe fluo-3-AM (Molecular Probes, Leiden). The intensity of fluorescence of fluo-3-AM increases in the presence of $Ca^{2+}$. Cell suspensions ($1.5 \times 10^8$ cells $ml^{-1}$, approximately 1 ml) were loaded with fluo-3-AM (final concentration 5 µmol $l^{-1}$) in 15 ml conical plastic tubes (light protected) at room temperature. The use of fluo-3-AM with its large fluorescence response may be a sensitive means for detecting destabilizing changes in sperm membranes (Harrison *et al.*, 1993). After 10 min of incubation, 4 ml HBS was added and the diluted suspension was incubated for an additional 20 min. This suspension was layered over 6 ml SWM and centrifuged as described by Harrison *et al.* (1993). The samples of bound, unbound and control spermatozoa were prepared from washed fluo-3-AM-loaded spermatozoa as described above. At each sampling point, the cell sample was placed on the slide, covered with the coverslip and placed on the stage of a Zeiss Axiovert 35 fluorescence microscope (Zeiss, Jena). The fluorescence was imaged using a camera (Hamamatsu 2400 SIT) linked to an Argus image processor system providing controlled opening of a shutter in the light path of the fluorescence excitation (described by Bicker, 1996). At each sampling point, a sequence of eight video images was taken, digitized as 256 × 256 pixels, and the pixel intensities were averaged. In > 95% of the sequences recorded, a total of 400 cells was evaluated in each category and at each sampling point (in the remaining 5% of records where the sperm concentration was too low, at least 50 cells were evaluated). For each cell, the relative corrected fluorescence $I_r = (I - I_0) / I_0$ was calculated from the data expressed in pixel intensities, where $I$ refers to the measured cell body (head and mid-piece) fluorescence intensity and $I_0$ represents the intensity of background fluorescence calculated as the mean of three intensity measurements on the places free of spermatozoa in each record field. The relative corrected fluorescence was averaged for all recorded cells.

### *Statistical analysis*

The means and standard errors of the mean were calculated for descriptive statistics. Multifactorial ANOVA was performed to analyse the effect of the cell state and the temporal effects on membrane characteristics, and thechi-squared test was used to examine the changes in the frequency distribution of the tyrosine phosphorylation patterns in spermatozoa. Regression analysis (linear regression) and non-linear modelling were used to describe the kinetic changes in terms of relationship between time of coincubation and parameters recorded. After unknown parameters of the model function fitting the data were estimated numerically, first derivatives were obtained from these by partial differentiation. The functions and their first derivatives were then compared pair-wise between cell samples (bound, unbound and TALP-incubated cell populations).

The Pearson correlation analysis was performed to determine the relationship between membrane integrity and motility.

Excel software and SAS Program Package (SAS Institute

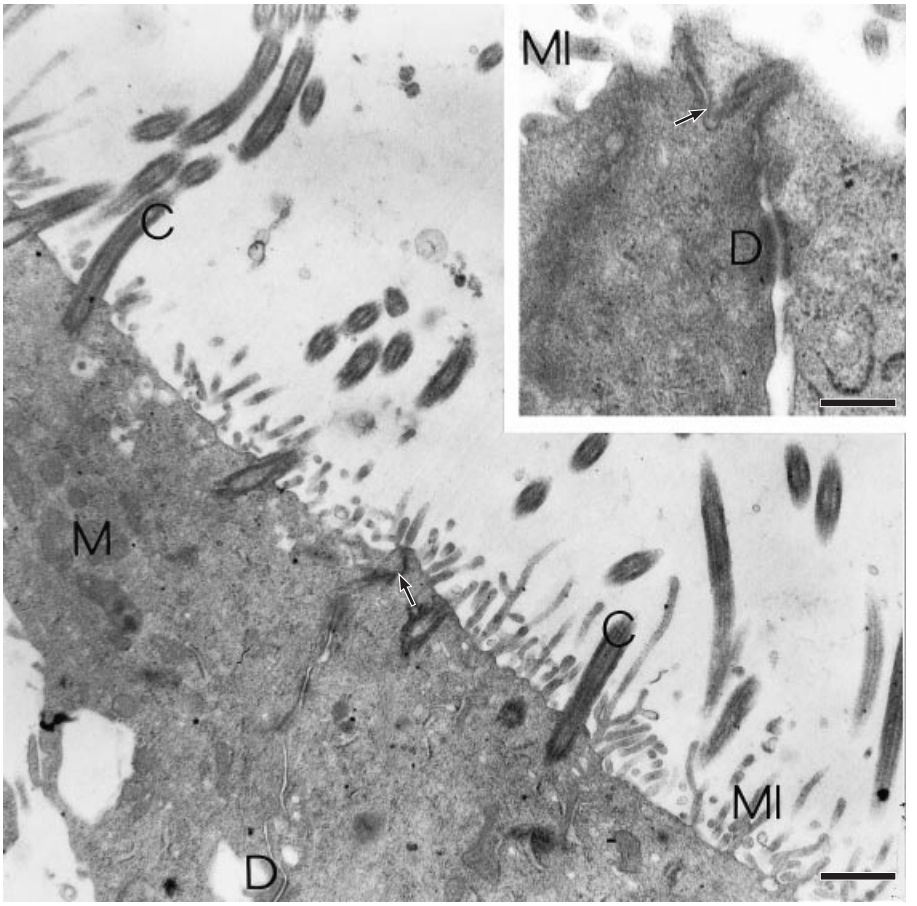


**Fig. 1.** Characterization of oviductal epithelial cell cultures by electron microscopy. Ciliated cells with cilia (C), microvilli (MI), mitochondria (M), tight junctions (arrow) with desmosomes (D) and widened intercellular space. Inset: higher magnification of the desmosomes (arrow) on the cell surface. The ciliated cells are characterized by apical cilia, microvilli and tight junctions between the cells, which are typical structures for epithelial cells. In other localizations of the tissue culture the intercellular spaces of the epithelial cells are widened markedly. The cytoplasm of the oviductal epithelial cells showed mitochondria, Golgi complexes, rough endoplasmic reticulum, some lysosomes and vacuoles. Scale bar represents 0.625 μm (main picture) or 0.25 μm (inset).

Inc., Cary, NC) were used for calculations in the above-described test procedures.

The level of significance was $P \leqslant 0.05$.

## Results

### *Immunohistochemical characterization of oviductal epithelial cell cultures*

The presence of the intermediate filaments cytokeratin (found in cells of epithelial origin only) and vimentin (found mainly in cells of mesenchymal origin but also in other developing cells like cell cultures) was demonstrated by immunocytochemistry to determine that the cultured cells were of epithelial origin. The antibody anti-pan-cytokeratin labelled filaments of oviductal epithelial cells in all cultured cells. The control samples, which were incubated with the primary antibody only, were not stained (data not shown).

### *Characterization of oviductal epithelial cell cultures by electron microscopy*

The light and electron microscope studies of the oviductal epithelial cells after 5 days of culture showed groups of ciliated cells of various sizes. In addition to ciliated cells, cells without ciliated surfaces were observed. The cell layer was covered by two epithelial cell types joined by tight junctions (Fig. 1). The ciliated cells were characterized by apical cilia and microvilli, and tight junctions between the cells, a typical structure for epithelial cells. In other areas of the tissue culture, the intercellular spaces of the epithelial cells were widened markedly.

Mitochondria, Golgi complexes, rough endoplasmic reticulum, some lysosomes and vacuoles were observed in the cytoplasm of the oviductal epithelial cells. Comparison of transmission electron microscope findings of the cultured oviductal epithelial cells and the normal oviductal epithelial cells in the oviduct revealed a good correlation. The morphology of the cells with normal nucleus, mitochondria and Golgi complex demonstrated viable epithelial cells.

The cells without cilia and microvilli were newly cultured cells after 5 days in culture, but are definitely epithelial cells, as demonstrated by the presence of tight junctions with desmosomes between the cells. Only a small number of fibroblast-like cells were observed in the cell layer on day 5 of culture.

### *Characterization of regulation of sperm functional changes*

Three sets of experiments were performed to investigate whether there is selection of spermatozoa by initial binding to oviductal epithelial cells and to study the possible regulation of sperm functional changes during coincubation. In Expt 1, the relationship between membrane integrity, coincubation time of spermatozoa and oviductal epithelial cells under capacitating conditions and status of spermatozoa (bound, unbound and TALP-incubated) was determined. In Expt 2, the relationship between tyrosine phosphorylation pattern, coincubation time and status of spermatozoa was studied. In Expt 3, the relationship between coincubation time, sperm cell status and intracellular $Ca^{2+}$ concentration was investigated. A series of preliminary experiments ($n = 5$) was also performed to ensure that incubation in TALP induces capacitation in boar spermatozoa. The capacitation state of boar spermatozoa was evaluated using chlortetracycline staining (essentially as described by Wang *et al.*, 1995). The mean percentage of uncapacitated spermatozoa decreased to 2.1% after 180 min of incubation compared with 55.4% at the beginning (3 min), whereas the mean proportions of capacitated and acrosome-reacted spermatozoa had increased after 180 min of incubation (82.2% and 16.0%, respectively, compared with initial values of 39.8% and 4.7%, respectively).

### *Changes in membrane integrity*

The membrane integrity of the TALP-incubated sample, measured using propidium iodide, yielded values of 12–37% dead spermatozoa (mean ± SEM: 21.3 ± 2.7%). After 3 min of coincubation, spermatozoa from these semen samples that bound to the oviductal epithelial cells showed the highest degree of membrane integrity (range 7–18%; 10.7 ± 1.1% dead spermatozoa). The lowest percentage of membrane-intact spermatozoa was observed in the unbound sperm preparation (37.6 ± 3.1% dead spermatozoa). The proportions of dead spermatozoa in the TALP-incubated semen samples and of spermatozoa bound to the oviductal epithelial cells were not related ($R^2 = -0.07$, $P = 0.82$).

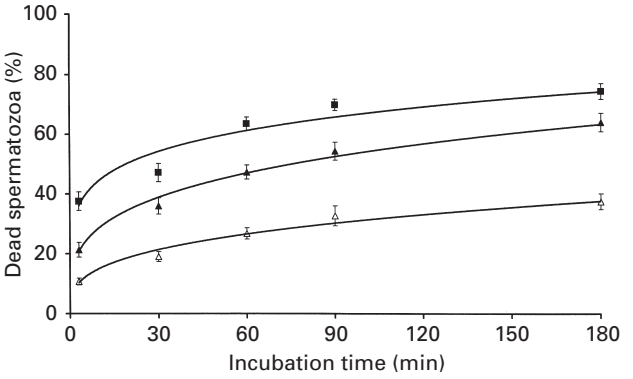


**Fig. 2.** Membrane integrity of spermatozoa bound to epithelial cells (△), unbound spermatozoa in co-culture with oviductal epithelial cells (■) and Tyrode's albumin lactate pyruvate (TALP)-incubated spermatozoa (▲) during incubation. Data are mean ± SEM ($n = 15$ ejaculates). The solid lines represent non-linear regression equations ($F(t)$) between time of incubation and the percentage of dead spermatozoa. $F(t) = 29.7\ t^{0.18}$ and $F'(t) = 5.35\ t^{-0.82}$ for unbound spermatozoa. $F(t) = 7.3\ t^{0.32}$ and $F'(t) = 2.34\ t^{-0.68}$ for bound spermatozoa. $F(t) = 15.4\ t^{0.27}$ and $F'(t) = 4.16\ t^{-0.73}$ for TALP-incubated spermatozoa. The cell death in the unbound sperm population proceeds at a faster rate than in spermatozoa selected by binding to oviductal epithelial cells: $F'(t)^{\text{unbound}} / F'(t)^{\text{bound}} = 2.29\ t^{-0.14} \geqslant 1$ for all $t \in \{3'; 180'\}$.

The ranking with respect to membrane integrity (unbound < TALP-incubated < bound) persisted at all sampling points, and the differences among the proportions of viable cells in these three populations were significant at all time points. The increase in the percentage of dead cells with the time of coincubation was observed in all three states (Fig. 2). Model equations fitting the data (see Fig. 2 legend) described these changes in viability as potential functions. Cell death progressed in approximately equal ratio in unbound and control sperm suspensions as shown by first derivative of the model function; however, the increase of cell death in bound cells was considerably delayed (Fig. 2 and Fig. 2 legend).

Changes in membrane integrity were accompanied by significant changes in sperm motility (Fig. 3). The motility decreased exponentially over the incubation period (model equations presented in Fig. 3 legend). The ranking with respect to motility was consistent with the ranking according to membrane integrity: unbound < TALP-incubated < bound spermatozoa. The slowest loss of motility was observed in bound spermatozoa (slower than in unbound within first 2 h of incubation; see Fig. 3 legend). At the end of incubation, 41.7 ± 4.3% of bound cells demonstrated intensive flagellar activity, whereas unbound spermatozoa were only 10.3 ± 1.5 % motile ($P < 0.05$).

Significant negative correlations were established between motility of sample and the proportion of dead spermatozoa in the cell population for bound, unbound and TALP-incubated cell samples (Pearson correlation coefficients $r = -0.78$, $-0.88$ and $-0.89$, respectively).

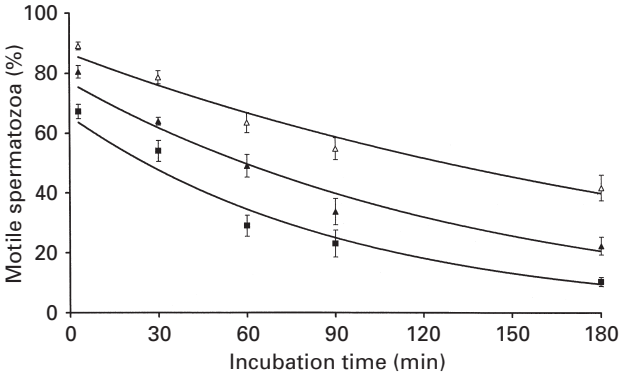


**Fig. 3.** Motility of spermatozoa bound to epithelial cells (△), unbound spermatozoa in co-culture with oviductal epithelial cells (■) and Tyrode's albumin lactate pyruvate (TALP)-incubated spermatozoa (▲) during incubation. Data are mean ± SEM ($n = 15$ ejaculates). The solid lines represent non-linear regression between time of incubation and the percentage of motile spermatozoa. $F(t) = 65.8\ e^{-0.011t}$ and $F'(t) = -0.72\ e^{-0.011t}$ for unbound spermatozoa. $F(t) = 86.6\ e^{-0.004t}$ and $F'(t) = -0.34\ e^{-0.004t}$ for bound spermatozoa. $F(t) = 77.1\ e^{-0.007t}$ and $F'(t) = 0.54\ e^{-0.007t}$ for TALP-incubated spermatozoa. The motility in the unbound sperm population decreases at a faster rate than in spermatozoa selected by binding to oviductal epithelial cells within the first 108 min: $F'(t)^{unbound}/F'(t)^{bound} = 2.12\ e^{-0.007t} \geqslant 1$ for all $t \in \{3'; 108'\}$; then $F'(t)^{unbound}/F'(t)^{bound} = 2.12\ e^{-0.007t} < 1$ for all $t \in \{108'; 180'\}$.

### *Tyrosine phosphorylation patterns*

Three main fluorescence patterns (differing within each in their intensity) were observed for unbound, bound and TALP-incubated spermatozoa. The bound spermatozoa showed either very faint 'dotted' fluorescence or were not fluorescent at all (referred to as pattern A). Two patterns of much higher intensity were observed in both free-swimming and TALP-incubated cell populations (Fig. 4). The pattern B (Fig. 4) of mid- or high intensity was characterized by the presence of a granulated ridge over the acrosomal region. The third pattern (C, Fig. 4), represents very intensive, almost homogeneous fluorescence in the acrosomal region.

No specific fluorescence was detected on the native cell cultures without spermatozoa. The samples incubated with first antibody inhibited by ortho-phosphotyrosine and the negative control samples incubated with the second antibody only did not show any specific fluorescence of the acrosomal region, only artefactual fluorescence in the post-equatorial segment (data not shown).

The proportion of cells showing pattern A was low in TALP-incubated spermatozoa (0–8%) and there were no cells without fluorescence. After coincubation of these sperm samples for 3 min with epithelial cells, most bound spermatozoa demonstrated fluorescence pattern A (60.4 ± 12.5%).

The distribution of fluorescence patterns was significantly different between cell populations selected by binding (bound and free-swimming) as well as between bound and TALP-incubated cells over all sampling points. No cells with patterns B or C were observed in spermatozoa bound on the cell cultures. The bound cells showed pattern A (64.8 ± 12.1%) or no fluorescence (35.2 ± 12.1%), whereas < 2% of the unbound or TALP-incubated cells showed this pattern during the whole period of incubation. Averaged over all sampling points, pattern C was observed most frequently in unbound spermatozoa (38.5 ± 11.7% of cells), whereas in TALP-incubated spermatozoa only 11.8 ± 7.8% of cells showed this fluorescence pattern. Pattern B was observed most frequently in TALP-incubated spermatozoa (86.5 ± 7.7%); the proportion of cells with this pattern among unbound cells was significantly lower (61.3 ± 11.9%).

The proportions of cells with pattern A in spermatozoa attached to oviductal epithelial cells and TALP-incubated sperm suspensions are shown (Fig. 5a). No significant temporal changes were observed. Within the first hour of incubation, no significant changes in tyrosine phosphorylation patterns B and C were observed in all populations; the first changes in the free-swimming cell population occurred after 90 min of coincubation of spermatozoa and oviductal epithelial cell cultures. Significant changes in the frequency distribution of patterns B and C between the start of incubation and after 180 min of incubation were observed in free-swimming and TALP-incubated spermatozoa (chi-squared test, Fig. 5b).

The non-linear models describing the changes in the proportion of cells with fluorescence pattern C are shown for free-swimming spermatozoa and TALP-incubated spermatozoa (temporal changes of pattern B are nearly complementary: the sum of proportion of cells with pattern B and C was close to 100% in unbound and TALP-incubated sperm populations; Fig. 5b). For unbound spermatozoa, the percentage of spermatozoa showing pattern C increased as a potential function of time and reached almost 250% of the initial value (52.2 ± 7.3%). The relative increase in the proportion of spermatozoa with pattern C was also observed in the control group; however, the changes and absolute response were lower in this group, and most of the population (85.2 ± 7.6% of cells) continued to express granulated pattern B.

### *Regulation of cytosolic $Ca^{2+}$ concentration*

At the time of first sampling, significant differences in the relative fluorescence intensity between unbound sperm samples ($I_r = 1.28$) and TALP-incubated/bound sperm samples were already observed ($I_r = 0.71$ and 0.47, respectively). The relative intensity ($I_r$) for $Ca^{2+}$ fluorescence was almost three times higher in unbound spermatozoa than in bound spermatozoa. The relative intensities in the TALP-incubated semen samples and in spermatozoa bound to the oviductal epithelial cells were unrelated. At the time of first sampling and after 60 min of coincubation, the

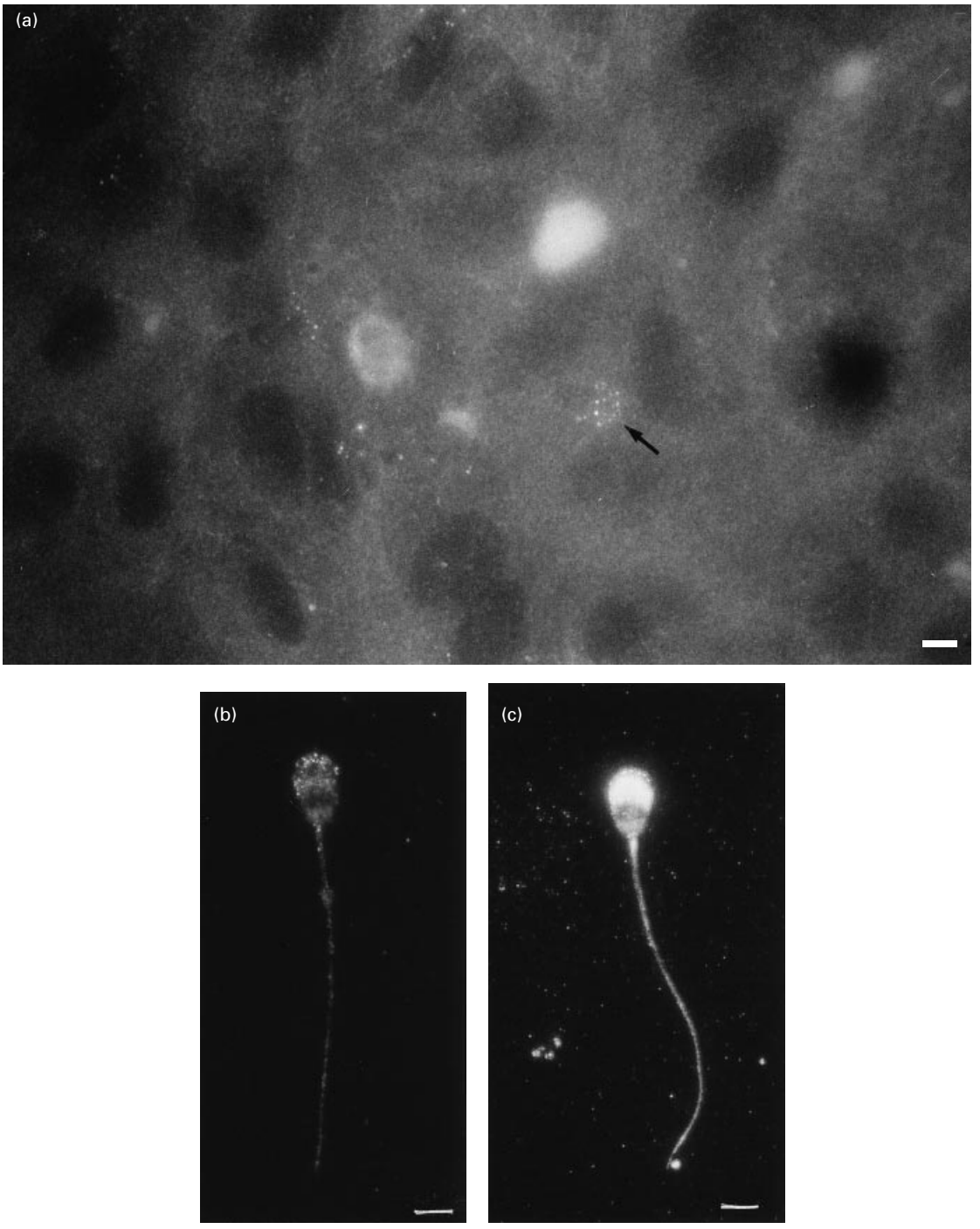


**Fig. 4.** Tyrosine phosphorylation patterns of spermatozoa (bound and unbound in co-culture with oviductal epithelial cells) and Tyrode's albumin lactate pyruvate (TALP)-incubated sperm suspensions. (a) Pattern A: very faint 'dotted' fluorescence localized in acrosomal region (arrow), or no fluorescence at all was observed in spermatozoa bound to the epithelial cells. (b) Pattern B: granulated pattern of medium to high intensity in acrosomal region, observed in unbound and TALP-incubated spermatozoa. (c) Pattern C: uniform pattern of very high intensity in acrosomal region, observed in unbound and TALP-incubated spermatozoa. Scale bars represent 5 μm.

fluorescence intensity in TALP-incubated sperm samples was significantly (about 50%) higher than in bound spermatozoa, but after 90 min of incubation this difference was no longer observed.

There was a considerable increase in cytosolic $Ca^{2+}$ concentrations in unbound spermatozoa during incubation (Fig. 6). The spermatozoa bound on the epithelial cell cultures showed very little increase in fluo-3-AM fluore-

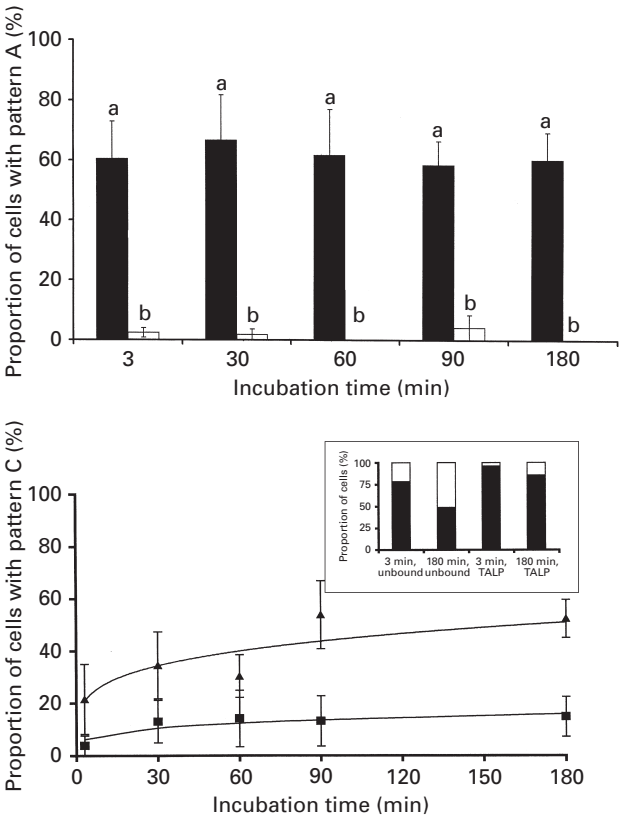


**Fig. 5.** Tyrosine phosphorylation patterns of spermatozoa during incubation. (a) Tyrosine phosphorylation pattern A of spermatozoa bound to epithelial cells (■) and Tyrode's albumin lactate pyruvate (TALP)-incubated spermatozoa (□) during incubation. $^{ab}$Different letters indicate significant differences between columns ($P < 0.05$). (b) Changes in tyrosine phosphorylation pattern C in unbound spermatozoa in co-culture with oviductal epithelial cells and control (TALP-incubated) spermatozoa during incubation. ▲: Proportions of cells with pattern C among free-swimming cells; and ■: proportions of cells with pattern C in TALP-incubated sperm suspensions. Solid lines represent non-linear regression between time of coincubation and the percentage of spermatozoa expressing pattern C. $F(t) = 15.07\ t^{0.24}$ and $F'(t) = 3.62\ t^{-0.76}$ for unbound spermatozoa. $F(t) = 4.82\ t^{0.23}$ and $F'(t) = 1.11\ t^{-0.77}$ for TALP-incubated spermatozoa. The increase in the proportion of cells showing intensive homogeneous fluorescence of the whole acrosomal region (pattern C) in the unbound sperm population proceeds at a faster rate than in spermatozoa incubated without oviductal epithelial cells: $F'(t)^{unbound}/F'(t)^{TALP} = 3.26\ t^{0.01} \geqslant 3$ for all $t \in \{3'; 180'\}$. Inset: the re-distribution of patterns B and C in unbound and TALP-incubated sperm populations during incubation. □: proportion of cells showing pattern C; and ■: proportion of cells showing pattern B.

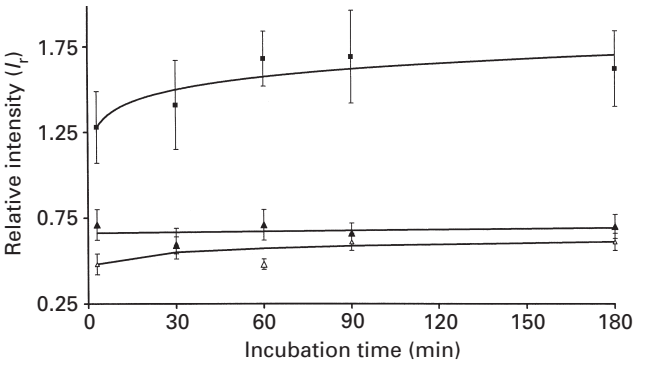


**Fig. 6.** Changes in $Ca^{2+}$ concentration of spermatozoa bound to epithelial cells (△), unbound spermatozoa in co-culture with oviductal epithelial cells (■) and Tyrode's albumin lactate pyruvate (TALP)-incubated spermatozoa (▲) during incubation, expressed as changes in relative intensity ($I_r$) of fluo-3-AM fluorescence. Solid lines represent non-linear regression between time of coincubation and the $I_r$ of fluorescence. $F(t) = 1.18\ t^{0.069}$ and $F'(t) = 0.081\ t^{-0.931}$ for unbound spermatozoa. $F(t) = 0.45\ t^{0.057}$ and $F'(t) = 0.025\ t^{-0.943}$ for bound spermatozoa. The relative intensity of fluo-3-fluorescence (reflecting cytosolic $Ca^{2+}$ concentration) increases at a faster rate in the unbound sperm population than in spermatozoa selected by binding to oviductal epithelial cells: $F'(t)^{unbound}/F'(t)^{bound} = 3.24\ t^{0.012} \geqslant 3$ for all $t \in \{3'; 180'\}$.

scence. The significant changes in mean $I_r$ were observed in both bound and free-swimming cell populations between 3 min and 90 min of incubation (difference $dI_r = I_r(90) - I_r(3) = 0.42 \pm 0.12$ and $0.13 \pm 0.06$ for free-swimming and bound cells, respectively). In free-swimming cells, the first significant changes were already observed after 60 min of incubation ($dI_r = 0.40 \pm 0.15$), whereas in bound spermatozoa the difference was not significant at this time. Model equations fitting the data (see Fig. 6 legend) described these changes as potential functions. Intensity increase was much greater (approximately 3.3–3.5 times greater during incubation period) in unbound sperm populations as shown by first derivative of the model function; the increase of cytosolic $[Ca^{2+}]_i$ in bound cells was delayed considerably (Fig. 6 and Fig. 6 legend).

The fluorescence intensity of TALP-incubated spermatozoa remained nearly unchanged.

There were large variations among individual donors with respect to the intensity of fluo-3-fluorescence and its temporal dynamics in unbound spermatozoa. Samples of some donors demonstrated maximum cytosolic $Ca^{2+}$ concentrations in unbound spermatozoa after only 30–60 min of coincubation; in samples from other boars, this maximum was not reached until after 90 or 180 min of incubation.

The possibility that the lower $Ca^{2+}$ concentration and low rate of fluo-3-positive cells were caused by selection of unloaded cells was eliminated by incubating the cells loaded with fluo-3-AM with the cell cultures for 3 and 60 min under the same conditions as in all experimental series, and 5 µl of 5 mmol calcium ionophore A 23187 $l^{-1}$ solution was added. In both bound and TALP-incubated cell suspensions, the addition of ionophore increased the fluorescence intensity ($I_r$) to 2.0–2.6 compared with the intensity in the absence of ionophore of 0.7–0.8, after only 3 min of (co)-incubation. The results after

60 min of (co)-incubation were essentially the same: after addition of ionophore the intensity increased to 1.9–2.3 compared with 0.7–0.8. The relative percentage of stained cells (fluorescence over gain threshold) increased from 19.6% and 17.5% in cells attached to oviductal epithelial cells and cells incubated in TALP, respectively, to 65% and 61.3%, respectively, after addition of ionophore after 3 min; similar results were observed after 60 min of incubation. As the possibility of preferential binding of cells with different fluorescent loading can be excluded, the differences in fluo-3-fluorescence may be interpreted as reflecting changes in $[Ca^{2+}]_i$ and, thus, the changes can be used for comparisons of different cell populations.

## Discussion

Capacitation is defined as the sequence of cellular and membrane alterations necessary to acquire fertilizing ability. The regulation of capacitation *in vivo,* which is mutually associated with the maintenance of viability, is a function of the oviduct. From various studies undertaken in different species it has been hypothesized that the binding of spermatozoa to the oviduct presents a mechanism for selecting a competent sperm population. Thomas *et al.* (1994) and Petrunkina *et al.* (2001) have reported that in equines and pigs, binding capacity depends on sperm quality, for example, morphology. Furthermore, spermatozoa bound to oviductal epithelium *in vitro* have intact acrosomes (Suarez *et al.*, 1991; Thomas and Ball, 1996), thereby indicating a selective function of sperm–oviduct binding. The studies in hamster and bull spermatozoa demonstrating higher binding ability of uncapacitated spermatozoa appear to point to the sperm capacitation status as a further criterion for selective interaction with the oviduct (Smith and Yanagimachi, 1991; Lefebvre and Suarez, 1996). The results of Dobrinski *et al.* (1996) assume the selectivity of binding to oviductal epithelial cells for equine spermatozoa with low $Ca^{2+}$ concentrations. Nevertheless, most of these studies lack strong evidence for the selectivity of initial binding due to a relatively long time period before first sampling (15–30 min). The strongest indication for preferential binding of uncapacitated boar spermatozoa to oviductal epithelial cells after a short incubation period was demonstrated by Fazeli *et al.* (1999). However, kinetic characterization allowing the rate of change initiated by contact with the epithelium to be estimated and to corroborate these conclusions was omitted. Furthermore, it is not currently known whether within some time period, cell populations selected by binding are destabilizing at a slower rate than non-selected spermatozoa.

In the present study, the hypothesis that the initial selection of boar spermatozoa with respect to their functional state takes place after only 3 min coincubation with oviductal epithelial cells was tested, and the kinetics of changes in membrane integrity and capacitation-related changes in tyrosine phosphorylation and cytosolic $[Ca^{2+}]_i$ were investigated. The initial functional state of cells bound to oviductal epithelial cells after 3 min of incubation was characterized by higher viability and lower $[Ca^{2+}]_i$ than in free-swimming spermatozoa. The absence of a correlation between the proportions of viable cells/cytosolic $Ca^{2+}$ concentrations of TALP-incubated samples (presenting functionally heterogeneous sperm populations) and those of spermatozoa bound to the epithelial cells provides evidence for the selectivity of initial binding to oviductal epithelial cells. No single spermatozoon with an enhanced phosphorylation pattern was found among bound spermatozoa, irrespective of the proportion of cells with the corresponding pattern observed in the free-swimming and TALP-incubated populations. Together, these observations strongly support the hypothesis that viable spermatozoa with low $Ca^{2+}$ concentration and suppressed tyrosine phosphorylation of membrane proteins attach preferentially to epithelial cells. It should be noted that no formal exact evidence for the selectivity of sperm binding to oviductal epithelium can be given using common oviductal epithelial cell methodology even if the spermatozoa are preincubated with the cultures for very short times. Theoretically, bound spermatozoa may undergo functional changes in the time between when the sperm suspensions are mixed with the cell culture and when they are processed for the first microscopic observation. However, given the kinetics of changes demonstrated in the present study, any potential influence of oviductal epithelial cells on spermatozoa within this very short interaction period (< 5 min) can be nearly ruled out.

During further long-term coincubation, oviductal epithelial cells appear to act to slow the process of sperm membrane destabilization and maintain cell viability and motility. Tyrosine phosphorylation was constantly low for the bound spermatozoa, whereas the free-swimming spermatozoa demonstrated rapid phosphorylation, as shown by immunofluorescence. Under capacitating conditions, differences between the functional state of bound and unbound spermatozoa were observed and the time courses of the processes have different kinetic models. A gradual increase in the percentage of dead cells in spermatozoa bound to oviductal epithelial cells was observed, although the magnitude of dynamic changes was about twofold lower than in unbound free-swimming cells. The relative intensity of fluo-3-fluorescence, which reflects changes in cytosolic $[Ca^{2+}]_i$, increased during the incubation at different rates in unbound and bound cell populations. This finding indicates that during a part of the period of attachment to the oviduct the viability is prolonged, the $[Ca^{2+}]_i$ increase is suppressed and destabilization of the plasma membrane/capacitation proceeds at a slower rate. Many investigators stress the role of direct contact between spermatozoa and oviductal epithelial cells for sperm survival (Smith and Yanagimachi, 1990; Smith and Nothnick, 1997) and for the delay of capacitation (Smith, 1998). However, the regulative role of the oviduct *in vivo* is not only to delay but also to promote capacitation to provide a sufficiently large population of

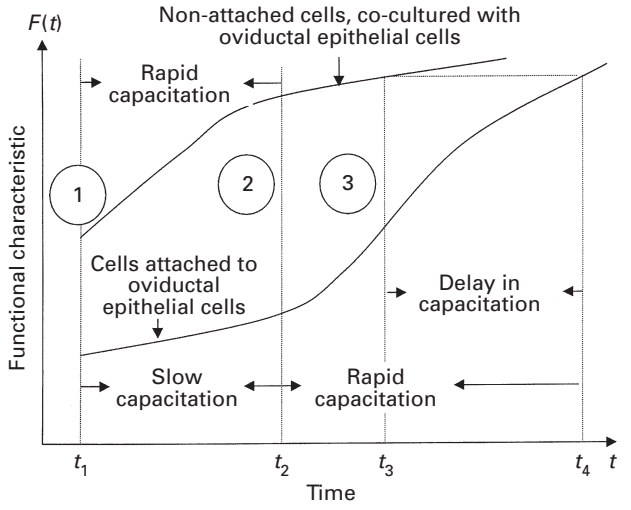


**Fig. 7.** Kinetic characterization of changes in sperm populations selected by binding to the oviduct. After initial contact with the oviductal epithelial cells the initial selective binding takes place according to differences in their functional characteristic $F$ (that may be a chosen parameter, such as proportion of capacitated cells assessed by chlortetracycline staining patterns or a set of parameters $F\{P_1, P_2,...,P_3\}$ describing the functional state of the sperm population). Functional characteristic $F$ of free-swimming cells is already greater than the functional characteristic of bound cells at this initial time point: $F^{free} > F^{bound}/(t_1)$. In physiological terms, uncapacitated viable morphologically normal cells with low intracellular $Ca^{2+}$ concentration are bound preferentially to oviductal epithelial cells. This event corresponds to the selective 'mode' (labelled as 1). During the following incubation period $\Delta t = t_2 - t_1$ the population of non-attached cells is destabilizing (capacitating?) more rapidly compared with the population selected by binding. The functional state of spermatozoa bound to oviductal epithelial cells is maintained or is changing slowly. This is characterized by conditions: $F^{free} > F^{bound}$ and $dF^{free}/dt > F^{bound}/dt$ for all $t \in \{t_1;t_2\}$. Afterwards, the process of functional changes accelerates, and destabilization/capacitation proceeds at a faster rate. The parametrical characteristics $F$ or $F\{P_1, P_2,...,P_3\}$ are increasing rapidly towards control values. Although within this time window $F^{bound}$ may still be $< F^{free}$ (capacitation delayed by $t = t_4 - t_3$), the cells attached to oviductal epithelial cells show rapid kinetics of changes: $F^{free} \geqslant F^{bound}$, whereas $dF^{free}/dt \leqslant F^{bound}/dt$ for $t > t_2$. The windows 2 and 3 both correspond to the 'regulative' mode; regulative processes taking place differ in their 'sign': in window 2, 'low' capacitation state would be maintained and the destabilization slowed; in window 3, the destabilization of attached cells proceeds at a fast rate.

cells capable of fertilization at the time of ovulation. In a number of studies performed *in vitro* and *in vivo* it has been demonstrated that capacitation and increase of cytosolic $Ca^{2+}$ concentration are involved in the release of spermatozoa from the epithelium (Smith and Yanagimachi, 1991; Lefebvre and Suarez, 1996; Dobrinski *et al.*, 1997), and that sperm capacitation proceeds at a faster rate when mating occurs after ovulation (Smith and Yanagimachi, 1989). Therefore, it can be expected that the interaction of the oviduct with spermatozoa occurs in three main modes: selection, maintenance of 'low capacitation' state within a certain time window and promotion of capacitation (Fig. 7). Under these circumstances, the kinetic characterization of functional changes of spermatozoa may be of special importance: comparison of cell proportions after certain periods of incubation could determine whether control levels are reached by this time. However, it would not determine whether the processes are decelerated or continue at the same rate and are just delayed due to primary selection. Kinetic characterization would make it possible to determine the rates and how much the 'delaying' or 'promoting' periods potentially important for investigating the factors determining capacitation rates *in vivo* are prolonged.

In conclusion, the results of the present study demonstrate that sperm–oviduct interaction in pigs is initiated by selective initial binding of spermatozoa to oviductal epithelial cells. Binding occurs preferentially to viable cells with low $[Ca^{2+}]_i$ and suppressed protein phosphorylation. The induction of changes in sperm functional state under capacitating conditions is slower in bound spermatozoa compared with free-swimming spermatozoa. The preferential initial binding and functional regulation of changes in boar spermatozoa attached to oviductal epithelial cells *in vitro* might represent a mechanism for selecting functionally competent spermatozoa and prolonging their lifespan by decelerating capacitation of spermatozoa stored in the sperm reservoir.

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