Redox control of surface protein sulphhydryls in bovine spermatozoa reversibly modulates sperm adhesion to the oviductal epithelium and capacitation

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Abstract

Oviductal fluid molecules, such as sulphated glycosaminoglycans and disulphide-reductants, may represent periovulatory signals for the release of spermatozoa from the oviducal reservoir in the bovine species. Disulphide-reductants release spermatozoa through the reduction of sperm-surface disulphides to sulphhydryls (SH). Herein, we studied sperm-surface protein SH through labelling with maleimidypropionyl biocytin in the initial sperm suspension, in the subpopulations able and unable to adhere to the in vitro cultured oviductal epithelium, and in spermatozoa released either through the disulphide-reductant penicillamine (PEN) or the sulphated glycosaminoglycan heparin (HEP). Adhesion assays were performed to study the ability of released spermatozoa to readhere to the oviductal epithelium. Results showed that the level of SH in sperm-surface proteins was: 1) low in adhering spermatozoa; 2) high in spermatozoa unable to adhere; and 3) markedly increased in released spermatozoa. Adhesion assays showed that: 1) PEN-released spermatozoa promptly recovered adhesion after removal of the disulphide-reductant and could be released again in response to PEN; 2) conversely, a limited number of HEP-released spermatozoa was able to readhere to the oviductal epithelium and this ability was not affected by HEP removal. Recovery of adhesion was associated to reoxidation of sperm-surface protein SH and to the reversal of capacitation. In conclusion, redox modulation of sperm-surface protein SH is involved in the release of spermatozoa adhering to the oviduct in vitro; the reversible action of disulphide-reductants might be responsible for intermittent phases of adhesions and releases; and the irreversible action of HEP indicates that it may represent a terminal releasing signal.


Introduction

In mammals, spermatozoa transiently adhere to the epithelial cells lining the caudal isthmus, the lower region of the oviduct, and this interaction extends the fertile life of spermatozoa in the female reproductive tract. The motility and capacitation of adhering spermatozoa remain suppressed for several hours, depending on the species, and reactivate 1 or 2 h before ovulation. At this time, discrete numbers of bound spermatozoa begin to be released and progress towards the ampullary/isthmic junction where the ovulated oocyte will arrest for fertilization. Increasing numbers of spermatozoa are activated and released during and even after ovulation, enhancing the numbers progressing towards the ampullary–isthmic junction (Hunter 2008). Hunter was the first to propose the term ‘functional sperm reservoir’ for the caudal isthmus (Hunter et al. 1980). Indeed, it is now well known, from in vivo and in vitro studies in different species, that the key mechanism able to select high-quality spermatozoa and preserve their fertile life is the adhesion itself (Dobrinski et al. 1997, Murray & Smith 1997, Smith & Nothnick 1997). Spermatozoa adhere to oviductal cell plasma membranes via molecules exposed on the sperm rostral surface capable of binding to carbohydrates on the oviductal cell surface, although the precise carbohydrates involved are species specific (DeMott et al. 1995, Dobrinski et al. 1996, Lefebvre et al. 1997, Green et al. 2001, Ignotz et al. 2001, Wagner et al. 2002, Cortés et al. 2004). In the cow, heparin (HEP)-binding proteins of the seminal plasma adsorb onto the epididymal sperm plasma membranes at ejaculation and are thought to promote sperm binding (Gwathmey et al. 2003, 2006) through their interaction with annexins 1, 2, 4 and 5 exposed on the apical surfaces of oviductal epithelial cells (Ignotz et al. 2007). On the other hand, far less is known about both the signals in the oviductal microenvironment, which promote sperm release and the sperm and/or oviductal cell surface

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molecules that mediate this release. We previously showed that sulphated glycoconjugates (Talevi & Gualtieri 2001) and disulphide-reductants (Talevi et al. 2007) are powerful inducers of the release of spermatozoa adhering to in vitro cultured oviductal epithelium. These two different classes of molecules have been suggested to represent physiological signals as they are similar to HEP-like glycosaminoglycans and reduced glutathione (GSH) respectively, both of which are present in the bovine oviductal fluid and increase in concentration at oestrus (Parrish et al. 1989a, Lapointe & Bilodeau 2003). Both release inducers were shown to act on spermatozoa and to trigger capacitation-related changes (Gualtieri et al. 2005, Talevi et al. 2007). Although the mechanisms of action of the two classes of inducers are only poorly known, sperm release induced by sulphated glycoconjugates has been shown to depend on sulphate groups. In fact, glycoconjugates that are devoid of sulphate groups, such as dextran (in contrast to dextran sulphate), and N-desulphated HEP, a glycosaminoglycan that fails to capacitate bovine spermatozoa (Miller & Ax 1989, Parrish et al. 1989b), also fail to trigger the release of spermatozoa adhering to the oviductal epithelium in vitro (Talevi & Gualtieri 2001). In the case of disulphide-reductants, we showed that oviductal adhesion selects spermatozoa devoid of surface sulphhydryls (SH) and that several permeant disulphide-reductants, as well as non-permeant disulphide-reductants (such as GSH), are able to release adhering spermatozoa by reducing surface disulphides (SS) to SH and by inducing capacitation (Talevi et al. 2007). Although we do not know the precise targets for the action of SS-reductants on the sperm surface, we hypothesized the existence of redox-sensitive surface proteins that directly or indirectly regulate the sperm ability to adhere to the oviductal epithelial cells.

In the present paper, we studied the SH groups of sperm membrane proteins exposed to the extracellular space through labelling with the impermeant maleimide derivative maleimidylpropionyl biocytin in the initial sperm suspension, in the subpopulation able and unable to adhere to the in vitro cultured oviductal epithelium and in spermatozoa released through either the SS-reductant penicillamine (PEN) or the sulphated glycosaminoglycan HEP. Adhesion assays were performed to study the ability of released spermatozoa to readhere to the oviductal epithelium. Results showed that 1) redox modulation of sperm-surface protein SS–SH regulates the ability of bovine spermatozoa to adhere to the oviductal epithelium; 2) both in vitro inducers of release act through the reduction of surface protein SS; 3) SS-reductants, and not HEP, represent a reversible signal for sperm release; and 4) recovery of sperm adhesion ability is associated with the oxidation of sperm-surface protein SH back to SS and the reversal of capacitation.

**Results**

**Effects of culture stage and BSA on sperm–oviduct binding and release**

To evaluate the effect of the oviductal culture stage on sperm binding and on HEP and PEN-mediated sperm release, in the present paper we analysed quantitatively the release of spermatozoa adhering to explants or monolayers. Explants bound spermatozoa at a higher extent compared with monolayers (Fig. 1A; P<0.05), but the response of spermatozoa adhering to the two oviductal culture stages to the inducers of release was similar. Data in Fig. 1A confirmed previous observational findings (Talevi & Gualtieri 2001, Talevi et al. 2007) demonstrating that PEN at 100 μM (Fig. 1A PEN) and HEP at 100 μg/ml (Fig. 1A HEP) is able to induce an almost complete release of spermatozoa adhering to both explants and monolayers compared with controls (Fig. 1A ADH; Fig. 1A, P<0.001).

In previous studies on sperm–oviduct binding and release (Talevi & Gualtieri 2001, Talevi et al. 2007), adhesion assays were carried out in Tyrode’s albumin lactate pyruvate medium (TALP) media containing 6 mg/ml BSA. However, such an amount of protein in media could affect maleimidylpropionyl biocytin (MPB) labelling and complicate the interpretation of data. Therefore, preliminary experiments (n=3) were carried out to evaluate whether the absence of BSA produced a non-specific sperm binding and/or affected sperm release. Data in

![Figure 1](image)
results obtained through ADH). These findings are in agreement with previous population (Fig. 2A UNB), whereas the lower level was detected in the unbound (UNB) sperm surface was detected in the unbound (UNB) sperm samples but the level of MPB labelling was markedly different. The higher level of SH exposed on the cell surface was detected in the unbound (UNB) sperm population (Fig. 2A UNB), whereas the lower level was detected in spermatozoa bound to monolayers (Fig. 2A ADH). These findings are in agreement with previous results obtained through in vivo labelling with iodoacetamide-fluorescein (IAF; Talevi et al. 2007) and show that in vitro adhesion to the oviductal epithelium selects spermatozoa endowed with surface proteins that expose a low level of SH. A second series of experiments (n = 3) was designed to understand whether PEN- and HEP-induced sperm release also affected MPB labelling of surface proteins. Comparison of adhering spermatozoa before and after release (Fig. 2B, ADH versus PEN-released spermatozoa (rPEN)) clearly shows that PEN caused the reduction of SS in several sperm-surface proteins. Several protein bands, barely visible in spermatozoa bound to monolayers, had an increased intensity after sperm release, whereas other protein bands that did not expose SH in spermatozoa bound to monolayers were reduced after sperm release (Fig. 2B, white triangles). The level of SH exposed in rPEN was quantitatively similar to that detected in the initial sperm suspension (Fig. 2B T0) although the pattern of proteins exposing SH was partially different, with some protein bands exposing more SH in rPEN than that in the initial sperm suspension and vice versa (Fig. 2B, black triangles). Analysis of MPB labelling in spermatozoa bound to monolayers and in spermatozoa released by HEP (Fig. 2C, ADH versus HEP-released spermatozoa (rHEP)) demonstrated that also this releasing agent causes a considerable SS reduction in most protein bands. Comparison between rPEN and rHEP (Fig. 2C rPEN versus rHEP) demonstrated a quantitatively similar SH exposition although qualitative differences among SH levels in corresponding protein bands were evident (Fig. 2C, white triangles). As described below, spermatozoa released by PEN quickly recover their adhesion ability upon removal of the inducer. Readhering spermatozoa had an MPB labelling pattern similar to that observed in spermatozoa bound to monolayers (not shown). In all experiments, analysis of parallel silver-stained gels demonstrated that the different extent of MPB labelling among samples was not due to a different protein loading. Figure 3A shows a representative silver-stained gel of the five sperm suspensions analysed. Comparison of protein patterns in silver-stained gels revealed only very slight differences among samples, such as the presence of a band of apparent molecular weight of 34 kDa that is more represented in rHEP (Figs 2C and 3A, black triangle). To rule out the possibility that the extent of MPB labelling was caused by a different percentage of dead and therefore permeabilized spermatozoa among samples, viability was assessed before MPB labelling. Data in Fig. 3B demonstrate that samples had not a significantly different viability except for the UNB versus the PEN-released subpopulation. Moreover, the low-protein sulphhydrylation pattern in spermatozoa bound to monolayers is not due to sequestration of MPB by the monolayers since surface SH groups were undetectable on oviductal cells (Talevi et al. 2007).

**Ability of released spermatozoa to readhere to the oviductal epithelium**

To assess the ability of released spermatozoa to readhere to the in vitro cultured oviductal epithelium, rPEN- and rHEP were added to monolayers at the same concentration used in the first adhesion assay in

![Figure 2](image)

**Figure 2** Western blots of sperm-surface proteins labelled with 3-(N-maleimidylpropionyl)biocytin (MPB). Adhesion selects spermatozoa with surface proteins exposing a low level of sulphydryls. (A) Comparison of MPB labelling among the initial sperm population (T0) and the unbound (UNB) and adhering (ADH) subpopulations. (B) In penicillamine-released spermatozoa (rPEN), the level of sulphydryls on sperm-surface proteins is increased. Black triangles point to protein bands that are differentially labelled in T0 versus rPEN. White triangles mark differences between rPEN and ADH. (C) In heparin-released spermatozoa (rHEP), the level of sulphydryls on sperm-surface proteins is increased. White triangles point to protein bands that are labelled more intensely with MPB in rPEN versus rHEP. Black triangle points to a protein band more represented in silver-stained gels of the rHEP subpopulation (Fig. 3A).
the presence or less of PEN 100 μM, or HEP 100 μg/ml, and incubated for 30 min (n=3). As shown in Fig. 4A, rHEP had a negligible ability to readhere and the extent of readhesion was not affected by the presence of the inducer in the medium (Fig. 4 A ADH W/O HEP, ADH W HEP). Conversely (Fig. 4 B), rPEN were able to readhere only after the removal of the inducer (Fig. 4B ADH W/O PEN versus ADH W PEN, P<0.001) and the ability to adhere was higher with respect to that shown by the initial population (P<0.01). Moreover, readhering spermatozoa were readily released again in response to 100 μM PEN (Fig. 4B, dashed bars ADH W/O PEN versus PEN, P<0.001) with an efficacy comparable with that shown by adhering spermatozoa.

**Labelling of SH groups of sperm surface with IAF**

To localize SH groups of sperm surface, aliquots of the same samples considered in the MPB labelling paragraph were labelled with the cell impermeant SH-labelling reagent IAF (n=4). As previously reported (Talevi et al. 2007), spermatozoa processed for localization of surface SH exhibited four labelling patterns, i.e. unlabelled (UNL), labelling of head (H), labelling of post-acrosomal region and tail (PA), labelling of the tail (T) (Fig. 5A). Data in Fig. 5B confirmed and extended previous results demonstrating that: 1) 62% of spermatozoa in the initial suspension were unlabelled; 2) 83% of adhering spermatozoa at 30 min of co-culture were unlabelled, showing that spermatozoa devoid of surface SH had a higher tendency to adhere to the oviduct (P<0.05); 3) 39% of spermatozoa unable to adhere were unlabelled (UNB versus ADH, P<0.001); and 4) the percentages of unlabelled spermatozoa significantly decreased after both PEN- and HEP-induced release (ADH versus rPEN or rHEP, P<0.01), and this was accompanied by a significant increase in the H pattern (P<0.001). Readhesion experiments showed that spermatozoa released by PEN quickly recover their adhesion
ability upon removal of the inducer. Spermatozoa recovering the adhesion ability showed a significant increase in the unlabelled pattern (rPEN versus READH, $P<0.001$) and a decrease in the H pattern compared with the rPEN ($P<0.001$), achieving values similar to those shown by adhering spermatozoa (Fig. 5C).

### Assessment of capacitation

Previous papers on the release of spermatozoa adhering to the oviductal epithelium *in vitro* have indicated that this event is due to changes in the sperm affinity towards the apical membrane of oviductal cells, and that such remodeling represents one of the earliest events of capacitation (Gualtieri & Talevi 2000, Talevi & Gualtieri 2001, Gualtieri *et al.* 2005). In particular, sperm release induced by thiol-reducing agents was associated with capacitation (Talevi *et al.* 2007). Here, we have shown that removal of PEN by released spermatozoa causes a rapid and massive recovery of the sperm-binding ability. To evaluate whether recovery of adhesion was associated with a reversal of capacitation, lysophosphatidylcholine (LPC)-induced acrosome reactions in the different sperm sub-populations were assessed with FITC-conjugated *Pisum sativum* agglutinin (PSA–FITC). Data (Fig. 6, $n=3$) confirmed that spermatozoa adhering to the oviductal epithelium are uncapacitated and that release induced by PEN was associated with a rapid change towards a capacitated state (ADH versus rPEN W, $P<0.001$). Interestingly, removal of PEN from released spermatozoa caused a reversal of capacitation (rPEN W versus rPEN W/O, $P<0.01$), whereas HEP removal did not affect the capacitation state. Viability assessments demonstrated that the extent of LPC-induced acrosome reactions was not due to a different percentage of viable cells among samples (viability %: rPEN W, $92.3\pm3.5$ versus rPEN W/O, $90.7\pm3.5$; rHEP W, $81.3\pm10.4$ versus rHEP W/O, $83.6\pm9$; $P=0.9$).

### Discussion

During the ascension through the female reproductive tract, only a small proportion of spermatozoa are able to overcome anatomical barriers such as the cervical mucus and the uterotubal junction. Spermatozoa entering the oviduct transiently adhere to the epithelial cells lining the isthmus, the lower region of the oviduct, and this event selects a high-quality sperm fraction and prolongs its life by delaying capacitation until, around ovulation, unknown signals induce the release of adhering spermatozoa (Smith & Yanagimachi 1991). Capacitation modulates the sperm ability to bind to and be released from the oviductal epithelium. In fact, only uncapacitated spermatozoa adhere to oviductal cells (Smith & Yanagimachi 1991, Leebvre & Suarez 1996).
and sperm detachment during the periovulatory period in vivo (Smith & Yanagimachi 1991), as well as the spontaneous release during in vitro co-culture (Gualtieri & Talevi 2000) are triggered by capacitation. The oviductal environment is a complex, regionalized and hormonally regulated milieu that is fine-tuned during the oestrous cycle to accomplish its different functions on gametes and embryos. In the bovine species, HEP-like glycoconjugates (Parrish et al. 1989a), as well as antioxidants, such as catalase and GSH (Lapointe et al. 1998, Lapointe & Bilodeau 2003) in oviduct luminal fluid, are under cyclic ovarian control. It is worth noting that they reach a peak during the period of oestrus, i.e. when spermatozoa selected and stored through the process of oviductal adhesion are released to migrate towards the fertilization site. Previous studies demonstrated that sulphated glycosaminoglycans and SS-reductants (such as GSH) powerfully trigger the release of spermatozoa adhering to the oviductal epithelium cultured in vitro, hence they were suggested to represent the periovulatory signals for sperm release in the bovine species (Talevi & Gualtieri 2001, Talevi et al. 2007). In particular, we observed that permeant as well as impermeant SS-reductants were powerful inducers of sperm release, their action was exerted on spermatozoa, and sperm release was associated with the reduction of sperm surface SS to SH. On the basis of these data, we demonstrated that redox regulation of SS–SH status of sperm surface modulates adhesion to the in vitro cultured oviductal epithelium (Talevi et al. 2007). Herein, we studied redox-sensitive surface proteins that could regulate the sperm ability to adhere to the oviductal epithelial cells. Main results showed for the first time that: 1) redox modulation of sperm-surface proteins regulates the ability of bovine spermatozoa to adhere to the oviductal epithelium; 2) both in vitro inducers of sperm release act through the reduction of surface protein SS; 3) SS-reductants, but not HEP, represent a reversible signal for sperm release; 4) recovery of the sperm adhesion ability is associated with the oxidation of sperm-surface protein SH back to SS and to the reversal of capacitation.

In vitro sperm–oviduct interaction has been studied in several different species using two different oviductal culture stages: freshly collected explants or in vitro cultured confluent monolayers. Although culture of explants until monolayer formation is accompanied by some signs of de-differentiation, such as the regression of cilia on the cell apical surfaces and the decrease in the cell height, previous papers demonstrated that monolayers retain several important features expressed by explants, as well as by the oviduct in vivo (Gualtieri & Talevi 2000, Sostaric et al. 2008). In the cow, monolayers are able to bind spermatozoa, to maintain their motility far longer than in UNB spermatozoa, and to depress their capacitation (Gualtieri & Talevi 2000, Gualtieri et al. 2005). In the present paper, HEP and PEN release spermatozoa from monolayers and explants with the same efficacy, further validating the use of monolayers as an in vitro model to study sperm–oviduct interaction. Analysis of the MPB-labelled proteins on western blots demonstrated that the oviduct cultured in vitro selects spermatozoa whose surface proteins have a low level of SH and, conversely, spermatozoa unable to adhere have a protein sulphhydration pattern similar to, but more heavily labelled than, the initial sperm suspension. IAF analysis confirmed MPB data and showed that the adhering subpopulation was primarily composed of spermatozoa devoid of surface SH, and of spermatozoa without detectable SH on the sperm rostral region that is involved in binding to the oviductal epithelium (Gualtieri & Talevi 2000). PEN-and, surprisingly, also HEP-induced sperm release were associated with a marked SS reduction in several protein bands. However, only a few had a different intensity when comparing sperm released with the two inducers. To our knowledge, this is the first study showing that HEP treatment is associated with the reduction of sperm-surface proteins. Parallel silver-stained gels and assessments of sperm viability demonstrated that the different extent of protein sulphhydration among samples was not related either to an unequal protein loading or to a different percentage of dead and permeabilized spermatozoa that could have led to an MPB labelling of intracellular proteins. IAF data were consistent with MPB findings showing a considerable and significant decrease in the unlabelled pattern and an increase in the H pattern after release with both inducers.

Recently, reversible redox modifications have emerged as physiological mechanisms for post-translational modulation of protein functions. SH–SS exchange plays a key role in the redox regulation of cell signalling and gene expression (Le et al. 2000, Pomposiello & Demple 2001, Hogg 2003, Barford 2004). Moreover, also
extracellular SH–SS exchange has recently been recognized as a way to regulate protein function. For example, SH–SS exchange modulates adhesion/aggregation of platelets through conversion of the receptor, integrin \( \alpha_{IIb}\beta_3 \), from a low- to a high-affinity state, and the interaction of CD4 receptor with the HIV-1 envelope glycoprotein gp120 to promote virus-cell fusion (Jordan et al. 1992). Therefore, the affinity of spermatozoa for the oviductal epithelium may be modulated by conformational changes driven by SH–SS exchange of specific sperm-surface proteins directly or indirectly involved in adhesion. The identification of those particular surface proteins is complicated by the presence of numerous reduced proteins that, according to IAF data, could be localized in sperm domains not involved in binding.

The extracellular environment, in contrast with the highly reducing nature of the intracellular compartments, is strongly oxidizing and contains proteins rich in SS bonds. As SH–SS exchange is a quickly reversible reaction, we designed experiments to understand whether removal of the reducing agent could lead to the oxidation of surface SH of released spermatozoa back to SS and cause a recovery of the sperm adhesion ability. Readhesion experiments clearly demonstrated that the removal of the SS-reductant from released spermatozoa causes an immediate recovery of the sperm adhesion ability. Oxidation of surface SH back to SS was confirmed by MPB and IAF data on readhering spermatozoa. To our knowledge, these data show for the first time that redox modulation of sperm-surface proteins may represent a reversible switch that would potentially allow an intermittent path of adhesions and releases during the sperm ascension through the female reproductive tract. Although, in the bovine, there is a lack of information in this respect, the possibility that the sperm ascent through the mammalian oviduct is intermittent is suggested by in vivo observations in favourable animal models. In fact, oviduct transillumination studies in naturally mated small rodents, such as mice and hamsters, showed that the typical pattern of sperm progression involves intermittent phases of adhesions and releases in response to local secretions of GSH, and of irreversible release in response to HEP-like glycosaminoglycans. Therefore, SS-reductants and HEP are able to fine-tune the adhesion, capacitation and release of spermatozoa in the oviduct, at least in vitro. A full comprehension of how these mechanisms operate in vivo would require a detailed knowledge of the different oviductal microenvironments that spermatozoa encounter during their ascension. During the oestrus cycle, spermatozoa adhering to the oviductal cells are likely to be exposed to much higher and variable concentrations of oviductal secretions compared with what can be appreciated by studies on macroenvironments such as the whole ampullar and isthmic fluids (Parrish et al. 1989a, Lapointe & Bilodeau 2003, Bergqvist & Rodríguez-Martínez 2006). Moreover, gametes are able to modulate their own microenvironment directly altering the oviductal secretory proteome (Georgiou et al. 2007). In particular, in pigs, spermatozoa have a direct influence on oviductal redox pathways, downregulating superoxide dismutase and phospholipid–hydroperoxide GSH peroxidase and upregulating thioredoxin (Georgiou et al. 2005).

In this context, it can be speculated that bound spermatozoa could be directly influenced by GSH secretions of neighbouring oviducal cells undergoing capacitation and release. Spermatozoa may travel a short distance reaching oxidizing microenvironments with decreased GSH concentrations, where sperm-surface protein SH can be promptly oxidized back to SS and this might be accompanied by the reversal of capacitation and recovery of the adhesion ability. As ovulation approaches, adhering spermatozoa might be exposed to local increases in HEP-like glycosaminoglycans that lead to their ‘terminal release’ and progression towards the ampulla.
Materials and Methods

Chemicals

BSA (fraction V), PEN, HEP (sodium salt, purified from porcine intestinal mucosa; H3393), LPC, 5-IAF, PSA–FITC, Hoechst 33342, protease inhibitor cocktail (P2714) and Medium 199 (M4530), were from Sigma Chemical Company; 3-N-MPB, FCS, gentamycin, amphotericin B, HEPES and sodium bicarbonate were from Invitrogen; glutaraldehyde was from TAAB Laboratories (Rome, Italy). Reagents and buffers for SDS-PAGE, including the molecular-weight Precision Plus Protein Standards, were from Bio-Rad. ImmunoPure Standard ABC Staining Kit and BCA Protein Assay Kit were from Pierce (Milan, Italy). Nitrocellulose (0.45 µm pore size; PROTRAN) was from Whatman (Dassel, Germany). Reagents and water for the preparation of salines and culture media were all cell culture tested.

Preparation of oviductal epithelial cells

Oviducts were collected at the time of slaughter and transported to the Laboratory in Dulbecco’s PBS supplemented with 50 µg/ml gentamycin at 4 °C. Laminae of epithelial cells were recovered from oviducts of single animals by squeezing and cultured at 39 °C, 5% CO2 in air, 95% humidity, in M199 supplemented with 50 µg/ml gentamycin, 1 µg/ml amphotericin B and 10% FCS. Bovine oviductal epithelial cells were either used as swimming-everted vesicles, referred to as explants, within 24 h of culture, or as confluent monolayers. To this end, explants were initially cultured in 10 cm Petri dishes (Falcon; Becton Dickinson, Milan, Italy) for 24–48 h and then transferred into four-well tissue culture plates (Nunclon, Roskilde, Denmark) with 12 mm, gelatin-coated, German glass round cover slips on the well bottom or on gelatine-coated 10 cm Petri dishes. Fresh media changes were performed every 48 h. Cell confluence was attained in about 7–10 days and monolayers were used within 24 h after attainment of cell confluence. Within each experiment, oviductal monolayers from a single individual were washed thrice in TALP (Parrish et al. 1989a), modified as described in Paula-Lopes et al. (1998), and left in this medium until sperm addition (0.5–1 h).

Sperm preparation

Frozen bovine semen from three bulls (0.5 ml straws; 3 × 108 spermatozoa per straw; motility after thawing ≥70%), obtained from Semen Italy (San Giuliano Saliceta, Modena, Italy), were used in all experiments. Straws were thawed in a water bath at 38 °C for 30 s and 0.5 ml semen was either labelled with 10 µg/ml Hoechst 33342 for 5 min at 39 °C, 5% CO2 in air, or directly washed in 10 ml and then in 5 ml TALP with or without BSA (BSA-free TALP), by centrifugation at 170 g for 10 and 5 min respectively. After resuspension in fresh medium, recovered spermatozoa were assessed for concentration and percent motility using a haemocytometer placed on a microscope stage heated to 39 °C.

Sperm–oviduct binding assays

Sperm suspensions recovered after centrifugation in TALP were added to monolayers cultured on gelatine-coated, 12 mm, round cover slips in four-well plates in 750 µl TALP at a concentration of 1–1.5 × 106 motile sperm/well, and incubated at 39 °C, 5% CO2 in air, 95% humidity, for 30 min. At the end of co-incubation, all free swimming spermatozoa were removed by extensive washings with TALP or collected for further analysis. This sperm fraction unable to adhere after 30 min of co-culture was referred to as ‘UNB spermatozoa’. After removal of UNB spermatozoa, monolayers with bound spermatozoa were left in TALP as controls, whereas parallel co-cultures were treated for sperm release. For sperm release, co-cultures were treated for 30 min with freshly prepared 100 µM PEN or with 100 µg/ml HEP. At the end of treatment, released spermatozoa were collected and sperm–oviductal co-cultures were fixed and analysed to quantify the number of adhering spermatozoa, as previously described (Gualtieri & Talevi 2000). Briefly, monolayers grown on gelatin-coated cover slips, inseminated with Hoechst-labelled spermatozoa, were fixed in glutaraldehyde 2.5% in PBS, for 1 h at 20–25 °C, extensively washed and mounted with the same buffer on a glass slide with cells facing up. For each well, fields of 0.0873 mm2 were acquired at a Nikon Eclipse TE 2000 inverted microscope under fluorescence, by means of a cooled camera head Nikon DS5 MC and NIS AR software (Nikon, Florence, Italy). The number of adhering spermatozoa was determined analysing ten fields of 0.0873 mm2 for each well.

A first series of experiments (n=3) was carried out to compare the ability of HEP and PEN to induce the release of spermatozoa bound to monolayers and to explants. Explants were inseminated in four-well plates in 750 µl TALP at a concentration of 1–1.5 × 106 motile sperm/well, washed free of UNB spermatozoa through transfer in three 100 µl droplets of TALP, treated with 100 µM PEN or with 100 µg/ml HEP, washed as above, and fixed and analysed to quantify the number of adhering spermatozoa. Explants (10 µl) were mounted on glass slides, covered with 24×24 mm cover slips, and ten fields of 0.0873 mm2 were acquired for each treatment. Monolayers were inseminated with identical sperm suspensions and number of motile sperm/well used for explants and treated as described above.

To assess the ability of PEN- and of rHEP to readhere to monolayers, the latter were inseminated at 1–1.5 × 105 motile sperm/well, washed free of UNB spermatozoa at 30 min and treated with PEN or HEP as above. Within each experiment (n=3), control monolayers and co-cultures treated with the two inducers were fixed to quantify the number of bound spermatozoa and therefore, indirectly, the extent of sperm release. Released spermatozoa were centrifuged at 170 g for 10 min, and added to monolayers using the same number of motile sperm/well as in the first adhesion. Readhesion assays were performed for 30 min in the presence or less of 100 µM PEN or 100 µg/ml HEP. The residual concentrations of PEN or HEP in the readhesion assays without the inducer were <1 µM and <1 µg/ml respectively.

As described below, MPB-labelling experiments were performed omitting BSA from media. Therefore, preliminary experiments (n=3) were carried out to evaluate whether the
absence of BSA produced a non-specific sperm binding and/or affected sperm release. To this end, spermatozoa washed as described above in BSA-free TALP were inoculated on monolayers with or without BSA, co-incubated for 30 min and washed free of UNB spermatozoa. Then, co-cultures with or without BSA were treated with PEN or with HEP as above. At the end of treatment, co-cultures were fixed to quantify the number of adhering spermatozoa.

**Labelling of sperm-surface proteins with MPB**

To study the sperm-surface proteins that exposed SH groups, sperm suspensions were labelled with the cell impermeant maleimide derivative MPB (de Lamirande & Gagnon 1998, 2003). Maleimides are alkylating agents that react with SH groups on cysteine residues to form stable carbon–sulphur bonds (Choi et al. 2007). All the sperm preparations were carried in BSA-free TALP and sperm–oviduct adhesion was performed using monolayers cultured on gelatine-coated 10 cm Petri dishes.

Sperm samples labelled with MPB included: 1) the initial sperm suspension; 2) bound and UNB spermatozoa after 30 min co-culture; 3) spermatozoa released by PEN or HEP and washed through centrifugation to reach a final concentration of the inducer in the MPB labelling mixture of 1 μM PEN or 1 μg/ml HEP respectively; 4) spermatozoa released by PEN and re-adhering to the oviductal epithelium after 30 min co-incubation (PEN residual concentration, 1 μM).

All sperm suspensions (n=3) were labelled with MPB 1 mM in 1 ml BSA-free TALP for 30 min at 39 °C, 5% CO2 in air, 95% humidity. After MPB labelling, sperm suspensions were diluted tenfold with BSA-free TALP, centrifuged 10 min at 170 g, resuspended in 5 ml 50 mM Tris–HCl (pH 7.4), containing 0.5% protease inhibitor cocktail, and centrifuged 10 min at 1000 g. Resulting pellets were treated 1:1 for 30 min on ice with extraction buffer (50 mM Tris (pH 7.4); 2 mM EDTA; 0.4% ‘Triton’ X-100; 0.5% protease inhibitor cocktail), centrifuged at 13 000 g, 5 min at 4 °C and the supernatants containing the labelled Triton-soluble fractions were processed for electrophoresis and western blotting as described below. Spermatozoa bound to monolayers cultured on gelatine-coated 10 cm Petri dishes were labelled with MPB 1 mM in 8 ml BSA-free TALP as above, extensively washed to remove the excess of MPB and then released by a 30-min treatment with 100 μM PEN. Resulting labelled suspensions were treated as above. The viability of all sperm suspensions before MPB labelling was assessed through the eosin/nigrosin test (Tartaglione & Ritta 2004). To allow a better visualization of viable cells in co-cultures, adhering and re-adhering spermatozoa were incubated in propidium iodide 25 μg/ml in BSA-free TALP for 15 min at 39 °C, 5% CO2 in air, 95% humidity, extensively washed with BSA-free TALP and assessed for viability using a fluorescence microscope.

**SDS-PAGE and western blot analysis**

Total proteins in supernatants containing the labelled Triton-soluble fractions were read by BCA Protein Assay Kit (Pierce). Sperm samples were treated for 30 min at room temperature in a standard Laemmli (pH 6.8) non-reducing buffer and then boiled for 5 min. For SDS-PAGE, an equivalent of 200 000 spermatozoa extracted in sample buffer (2 μg) was loaded on 10 or 12% gels with a 3% stacking gel. In all experiments, two parallel gels were prepared, one was electrophoresed to a 0.45 μm pore nitrocellulose membrane and the other was silver-stained to ascertain that the same amount of proteins was applied. Detection of MPB-labelled protein bands was accomplished by overlaying the blots with HRP-conjugated streptavidin (ImmunoPure ABC Standard Peroxidase Staining Kit; Pierce), which bind to the biocytin portion of MPB, and then with 3-amino-9-ethyl carbazole (Sigma) according to the manufacturer’s instructions.

**Immunofluorescent labelling of sperm-surface SH**

To study the sperm-surface SH distribution patterns, aliquots of the same samples considered in the MPB labelling paragraph were labelled with IAF (n=4) as previously described (Talevi et al. 2007). Briefly, 100 μl aliquots of sperm suspensions in TALP were incubated with 4 μM IAF for 15 min at room temperature in the darkness, diluted to 10 ml with TALP, centrifuged for 10 min at 170 g, resuspended in fresh medium and observed in vivo using a fluorescence microscope. Monolayers grown on gelatin-coated cover slips with adhering spermatozoa were labelled with IAF as above, extensively washed with TALP, mounted with fresh medium on a glass slide with cells facing up and observed in vivo using a fluorescence microscope. Ten to twenty fields (depending on sperm concentration) were alternatively acquired with bright field and fluorescence imaging. Labelling patterns were expressed as percentages through analysis of at least 500 spermatozoa for each condition.

**Assessment of capacitation**

The sperm capacitation status was studied through assessment of LPC-induced acrosome reaction (Talevi et al. 2007). To this end, sperm suspensions or co-cultures treated with 100 μg/ml LPC (stock solution, 30 mg/ml in ethanol) or with 0.33% ethanol (control) in TALP for 15 min were placed on glass slides and air dried. Samples were then processed for assessment of acrosomal status with PSA–FITC as follows: samples were permeabilized in 95% ethanol for 1 h, washed in PBS, incubated for 30 min in the darkness with 10 μg/ml PSA–FITC (Sigma) in PBS, washed extensively in PBS, mounted and observed using a fluorescence microscope. Fields were alternatively acquired under phase contrast and fluorescence. Acrosomal status was evaluated by scoring at least 500 spermatozoa treated or not with LPC for each condition. Spermatozoa with a brightly fluorescent acrosomal cap were considered acrosome-intact. Capacitation of a given sperm population was determined by the difference between percentages of acrosome-reacted spermatozoa in LPC-treated and in basal populations. To understand whether recovery of adhesion ability of released spermatozoa was associated to a reversal of capacitation, released spermatozoa were centrifuged and incubated in control or LPC media in the presence or
less of 100 μM PEN or 100 μg/ml HEP. The residual concentrations of PEN or HEP in control or LPC media without the inducer were 1 μM and 1 μg/ml respectively. Viability of released spermatozoa after incubation in LPC media was assessed as described above.

**Statistical analysis**

The data are presented as mean±S.D. Overall analysis was performed by the estimate model of ANOVA (SAS/STAT 1988) followed by the Tukey’s honest significant difference test for pairwise comparisons when overall significance was detected. Results expressed as percentages were modified by arcsine transformation to normalize the data.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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