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Original Article

Protein tyrosine phosphorylation of the human sperm head during capacitation: immunolocalization and relationship with acquisition of sperm-fertilizing ability

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Abstract

The occurrence of tyrosine phosphorylation (TP) in the sperm head during capacitation has been poorly investigated, and no data exist on the relationship of its dynamics with the acquisition of sperm fertilizing ability. This study localized TP of head proteins in human spermatozoa during capacitation and explored its relationship with acquisition of the ability to display progesterone (P)-stimulated acrosome reactions (ARs) and to penetrate zona-free hamster oocytes. By immunofluorescence, TP immunoreactivity was revealed in the acrosomal region of formaldehyde-fixed/unpermeabilized samples, whereas it was abolished in fixed/permeabilized samples, in which TP immunoreactivity was high in the principal piece. No TP immunoreactivity was detectable in unfixed spermatozoa. Head TP immunoreactivity was localized externally to the acrosome, close to the cytoplasmic membrane, as assessed by transmission electron microscopy. The increase in head TP was an early event during capacitation, occurring within 1 h in capacitating conditions. At this time, the P-stimulated ARs were also increased, whereas egg penetration was as poor as in uncapacitated spermatozoa. At 5 h of capacitation, the extent of neither head TP nor the P-induced ARs were greater than that at 1 h, whereas egg penetration had significantly increased. Seminal plasma inhibited head TP, P-induced ARs and egg penetration. None of these inhibitory effects, unlike those on tail TP, were prevented by the cAMP analogue dbcAMP (*N*,2-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate). In conclusion, head TP is a subsurface event occurring early during capacitation and is closely related to acquisition of the ability to display P-stimulated ARs, whereas the ability to fuse with oolemma and to decondense is a later capacitation-related event.

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1 Introduction

Mammalian spermatozoa must undergo capacitation in the female reproductive tract to acquire the ability to fertilize oocytes. Capacitation enables spermatozoa to gain hyperactive motility, adhere to the zona pellucida,

respond to physiological inducers of the acrosome reaction (AR) and initiate fusion with the oocyte [1]. The increase in tyrosine phosphorylation (TP) of sperm proteins is a major event occurring during capacitation [2], and some studies have correlated the level of TP with the capacitated state of mammalian spermatozoa [3, 4]. Indeed, factors with a role in regulating capacitation also regulate TP. In particular, both sperm TP and capacitation are stimulated by cAMP analogues and phosphodiesterase inhibitors and are inhibited by protein kinase A (PKA) inhibitors, thereby suggesting that cAMP/PKA signalling pathways are involved in the two processes [5]. Furthermore, seminal plasma, which prevents capacitation [6], also prevents sperm TP [7]. Nevertheless, the suggestion that TP could be a marker of a fully capacitated state is contradicted by the observation of an increase in TP of a 32-kDa protein in porcine spermatozoa during incubation in non-capacitating media (depleted of bicarbonate [8] and under PKA pathway inhibition [9]).

In humans, the relationship between increase in TP and acquisition of sperm fertilizing ability during capacitation has been poorly investigated. In a recent study, we explored the relationship between the capacitation-related increase in global TP, quantified by a flow cytometric assay, and the acquisition of human sperm fertilizing ability, evaluated by the progesterone (P)-enhanced hamster egg penetration test (HEPT) [7]. An increase in global TP seemed to be an early event in the capacitation process, whereas the P-enhanced sperm–oocyte fusion required a longer capacitation time. Furthermore, it was possible to dissociate the increase in TP and the P-enhanced egg penetration under different experimental conditions, suggesting that sperm fertilizing ability is always associated with an increase in global TP, whereas TP does not necessarily reflect the acquisition of sperm fertilizing ability. However, global TP, quantified in fixed and permeabilized sperm suspensions, reflected the TP of sperm proteins that were mainly distributed along the flagellum, as evaluated by immunofluorescence. Indeed, most of the rare studies that have explored the link between the phosphorylation status of mammalian spermatozoa and their fertilizing ability have focused on TP of sperm flagellar proteins, because the flagellum seems to be the major sperm compartment undergoing TP in a number of species [10–15]. In particular, in human spermatozoa, PKA-anchoring proteins (AKAPs) localized on the fibrous sheath, namely, AKAP82, its precursor pro-AKAP82 and FSP95, are the most prominent tyrosine-phosphorylated proteins during capacitation [10, 14]. Although it has been reported that a low percentage of human spermatozoa with phosphotyrosine residues on the principal piece is associated with reduced sperm–zona pellucida binding [16] and reduced *in vitro* fertilization [17], the link between the increase in tail TP and the acquisition of hyperactivated motility is better established [11, 12, 14, 15].

Spermatozoa are highly polarized cells, with the head performing functions related to oocyte interaction and the tail being involved in energy production and motility. Therefore, the increase in TP of the flagellum could be related to the onset of hyperactivated motility during capacitation [11, 12], but it cannot directly account for the acquisition of the ability to interact with the oocyte, where the sperm head is involved.

A superficial TP immunoreactivity was reported in the head of live mouse spermatozoa by the use of immunomagnetic beads [18]. In that study, capacitation promoted the appearance of tyrosine-phosphorylated chaperone proteins on the sperm surface overlying the acrosome, which is thought to facilitate sperm–zona binding. Unfortunately, the same authors failed to confirm these results in humans [19]. Nevertheless, previous reports have described a reduction in zona pellucida binding [20] and sperm–oocyte fusion [21] when human spermatozoa were pre-incubated with monoclonal anti-phosphotyrosine antibodies. Scanty and conflicting results exist on the occurrence of or increase in subsurface head TP during capacitation [13, 17, 21–23].

In the present study, which used fixed un-permeabilized human sperm suspensions and immunofluorescence, we revealed a capacitation-dependent TP of head proteins that had a subsurface localization. We also explored the relationship between head TP and capacitation-dependent acquisition of the ability to undergo P-dependent ARs and egg penetration.

2 Materials and methods

The study was approved by the Ethics Committee of the Azienda Sanitaria Locale of L'Aquila and all subjects signed an informed consent statement.

2.1 Chemicals

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless stated otherwise. P was prepared as a stock solution in dimethyl sulfoxide (DMSO).

P, *N*,2-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP) and H89 were diluted in Biggers, Whitten and Wittingham (BWW) medium to give the final working concentration before use. *O*-phospho-*L*-tyrosine was diluted in NH_4OH (4 mol L^{-1}).

2.2 Sperm processing

Semen samples were collected according to the World Health Organization-recommended procedure [24] by masturbation from healthy normozoospermic donors. All samples were collected in sterile containers and left for at least 30 min to liquefy at 37 ºC before processing. Motile sperm suspensions were obtained by a swim up procedure. Briefly, spermatozoa were washed twice (700 × *g*, 7 min) in BWW medium (pH 7.4). After the second centrifugation, supernatants were removed by aspiration, leaving 0.5 mL on the pellet, and after 30 min the supernatants, containing highly concentrated motile spermatozoa, were carefully aspirated, and the sperm concentration was adjusted to 7×10^6 spermatozoa per mL. To evaluate the effect of seminal plasma, 50% seminal plasma/50% BWW medium (pH 7.4) (v/v) was used for washing, the swim up procedure and incubation in capacitating conditions. Seminal plasma was recovered by centrifugation (700 × *g*, 7 min) of ejaculates from five normozoospermic subjects, sterilized by filtration (Millex GV filters [0.22 μm], Millipore, Bedford, MA, USA), aliquoted, stored at −80 ºC and thawed before use. Motile sperm suspensions were incubated under capacitation conditions for 1 h and 5 h at 37 ºC in an atmosphere of 5% CO_2/95% air (v/v) in the presence of 1% (w/v) human serum albumin fraction V in the capacitation medium (BWW). Sperm viability, determined by eosin–nigrosin exclusion staining [24], remained unchanged during 5-h capacitation, and always exceeded 90%.

2.3 Immunocytochemistry

Immunocytochemistry was performed on (1) live motile sperm suspensions; (2) fixed unpermeabilized motile sperm suspensions; and (3) fixed and permeabilized motile sperm suspensions. Fixation was performed by adding either ice-cold 1% (v/v) formaldehyde in phosphate-buffered saline (PBS, pH 7.4) or ice-cold absolute methanol for 30 min at 4 ºC. Permeabilization of fixed spermatozoa was performed by using 0.1% (v/v) Triton X-100 in PBS for 10 min at room temperature (RT). Tyrosine phosphoproteins were recognized by a fluorescein isothiocyanate-labelled monoclonal antibody (mAb) against human phosphotyrosine (clone pY20). The optimal working concentration of the anti-phosphotyrosine mAb (10 μg mL^{-1}) and the time of co-incubation with spermatozoa (1 h) were chosen after preliminary experiments. To evaluate the immunostaining specificity, pY20 mAbs were pre-incubated with a saturated solution of 50 mmol L^{-1} *O*-phospho-*L*-tyrosine for 1 h at RT before their addition to motile sperm suspensions incubated under capacitation conditions for 5 h. After three centrifugations (700 × *g*, 7 min), sperm suspensions were smeared on slides, mounted in PBS–glycerol and observed with a fluorescence microscope (Leica DMLB, Wetzlar, Germany) at × 100 magnification.

2.4 Transmission electron microscopy

A pre-embedding procedure and a sensitive peroxidase system were used to detect the subcellular localization of tyrosine-phosphorylated sperm head proteins. Motile sperm suspensions incubated under capacitation conditions for 5 h were washed by centrifugation (1 000 × *g*, 4 min) in PBS (pH 7.2), and pellets were resuspended in 2.5% (v/v) glutaraldehyde (AGAR Scientific Ltd, Essex, UK) in sodium cacodylate buffer (pH 7.2), for 1 h at 4ºC. The immunostaining procedure was performed according to the protocol from the Envision + Dual Link System-HRP kit (DakoCytomation, Carpentaria, CA, USA). The high sensitivity of this system is based on a horseradish peroxidase-labelled polymer that is conjugated to the secondary antibodies. Briefly, after centrifugation, endogenous peroxidase was blocked with 0.3% (w/v) hydrogen peroxide containing sodium azide (1 mmol L^{-1}), and after further centrifugation as above, spermatozoa were incubated overnight at RT with pY20 mAbs or MOPC-21 mAbs as a control, both diluted in the ratio 1:50 in PBS with 2% (w/v) bovine serum albumin. After three centrifugations as above in PBS, pellets were resuspended in a peroxidase-labelled polymer conjugated to goat anti-mouse $F(ab')_2$ immunoglobulins for 1 h at RT, followed by a washing step in PBS. Immunoreactivity was revealed with 3′,3′-diaminobenzidine (DAB) as the chromogen. After repeated washes by centrifugation, samples were post-fixed in 1% (w/v) osmium tetroxide in distilled water, dehydrated in graded ethanol and embedded in Epon 812 (AGAR Scientific Ltd., Stansted, UK). Ultrathin sections (70 nm) were cut on a ultramicrotome (Reichert, Depew, NY, USA) equipped with a diamond knife, then contrasted briefly with uranyl acetate and lead citrate

(AGAR Scientific Ltd.) and viewed in a Philips CM100 transmission electron microscope (Philips Electronics, Eindhoven, the Netherlands).

2.5 Hamster egg penetration test

The P-enhanced HEPT was performed as previously described [7]. Briefly, motile sperm suspensions were incubated under capacitating conditions for 1 and 5 h and then exposed to P (5 μmol L^{-1}) for 15 min or to the same volume of DMSO (as a control) before incubating with oocytes.

In one set of experiments, spermatozoa treated with 50% (v/v) seminal plasma were incubated under capacitating conditions for 5 h in the presence or absence of the cell-permeable cAMP analogue dbcAMP (5 mmol L^{-1}) and then exposed to P (5 μmol L^{-1}) or DMSO before incubating with oocytes.

Standard procedures were used for the recruitment of oocytes from mature golden Syrian hamsters of the species *Mesocricetus auratus* [24]. Between 15 and 20 zona-free oocytes were added to each 100-μL droplet containing 0.7×10^6 motile spermatozoa. After 3 h co-incubation at 37 ºC in an atmosphere of 5% CO_2/95% air (v/v), oocytes were recovered from the droplets, washed free of loosely adherent spermatozoa and labelled with SYBR 14 (2 μmol L^{-1}). SYBR 14 is a nuclear membrane-permeable DNA-specific fluorochrome. It stains sperm nuclei and emits fluorescence at 515 nm. To determine sperm penetration, oocytes were examined for the evidence of swollen sperm heads in the cytoplasm with a microscope equipped with epifluorescence (Leica DMLB). The number of spermatozoa penetrating each egg was assessed and expressed as the total number of penetrations/total number of oocytes (penetration index).

2.6 AR assessment

Assessment of P (15 μmol L^{-1})-induced ARs was performed as previously described [25] in the same experimental conditions used for the HEPT. Briefly, sperm suspensions were centrifuged and resuspended in hypoosmotic solution for 1 h to check for sperm viability [24]. After centrifugation, sperm suspensions were smeared, fixed in methanol, incubated with fluoresceinated *Pisum sativum agglutinin* at 100 μg mL^{-1} in PBS (pH 7.2) for 2 h, washed and observed under a fluorescence microscope (Leica DMLB). At least 200 spermatozoa were counted in each smear, and the percentage of spermatozoa not uniformly fluorescing at the anterior region of the head (reacted spermatozoa) was evaluated. Only spermatozoa with coiled tails were considered viable and thus scored for true (non-degenerative) ARs [26]. P-induced ARs were calculated as increase in the AR rate after exposure to P.

2.7 Statistical analysis

Statistical analysis was performed using the SAS statistical software (version 9.1; SAS Institute Inc., Cary, NC, USA). Immunocytochemistry and AR data were analysed by analysis of variance (ANOVA). The HEPT results were subjected to two-way analysis of variance to separate replicate from treatment variations (general linear model procedure, PROC GLM). *Post hoc* comparisons between pairs of groups were performed using the Tukey's studentized range (HSD) test and statistical significance was set at $P \leq 0.05$. Results were expressed as mean ± SEM.

3 Results

3.1 Immunodetection of sperm head TP

Using immunofluorescence, head TP immunoreactivity was revealed only in formaldehyde-fixed/unpermeabilized samples. A fluorescent signal distributed in the anterior region of the head was observed for the majority of 5-h-capacitated spermatozoa (Figure 1A). A minority of spermatozoa also exhibited fluorescence along the principal piece of the sperm tail (Figure 1A). Both methanol fixation and permeabilization of formaldehyde-fixed sperm suspensions completely abolished head TP immunoreactivity, although they increased TP immunoreactivity of the principal piece (Figures 1B and C). No TP immunoreactivity could be detected in viable unfixed 5-h-capacitated spermatozoa (Figure 1D). Preincubation of the anti-phosphotyrosine mAb with a saturated solution of 50 mmol L^{-1} *O*-phospho-*L*-tyrosine, its specific antigen, abolished all TP immunoreactivity in formaldehyde-fixed/unpermeabilized (Figure 1E) and fixed/permeabilized 5-h-capacitated spermatozoa (Figure 1F).

Head TP immunoreactivity was localized externally to the acrosome, close to the cytoplasmic membrane, as assessed by transmission electron microscopy (Figure 2).

3.2 Time course of sperm head TP, ARs and oocyte penetration during capacitation

The increase in head TP, as evaluated in formaldehyde-fixed/unpermeabilized samples, seemed to be an

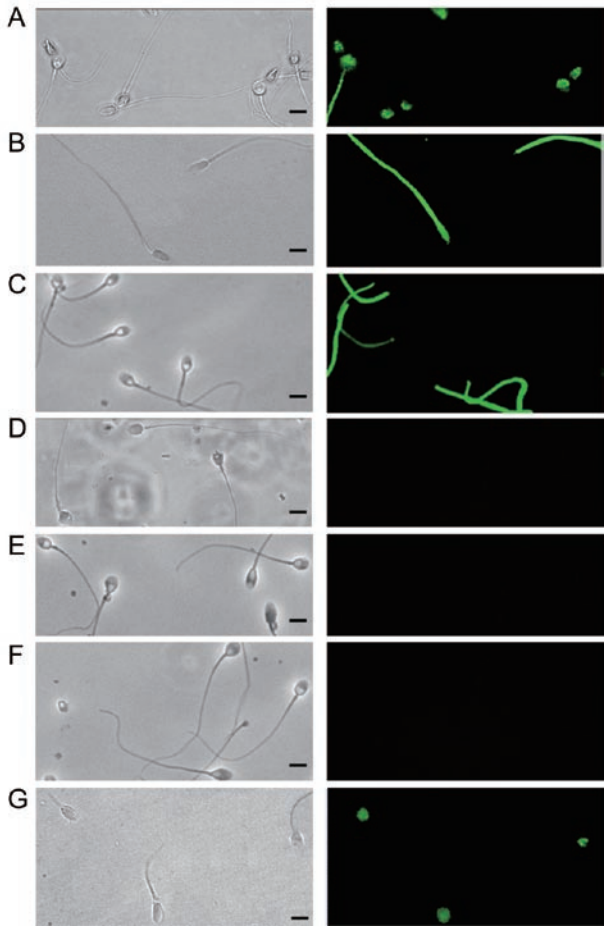

Figure 1. Immunoreactivity of monoclonal antibody pY20 by an immunofluorescence test on 5-h-capacitated human spermatozoa using (A) formaldehyde-fixed/unpermeabilized, (B) methanol-fixed and (C) formaldehyde-fixed/permeabilized spermatozoa. No fluorescent labelling was detected with unfixed spermatozoa (D). There was no fluorescent labelling when formaldehyde-fixed/unpermeabilized (E) and fixed/permeabilized (F) spermatozoa were exposed to pY20 mAb pre-incubated with a saturated solution of *O*-phospho-*L*-tyrosine. (G): Immunoreactivity of formaldehyde-fixed/unpermeabilized spermatozoa was unchanged after 5 h of incubation under capacitating conditions in the presence of the protein kinase A inhibitor H89 (50 μmmol L^{-1}). Phase contrast (left) and corresponding immunofluorescence photographs (right) (Bars = 5 μm).

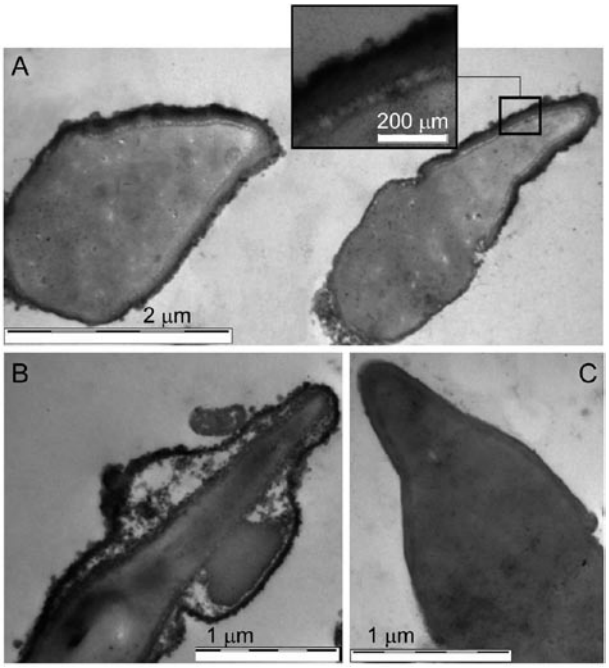

Figure 2. Transmission electron micrographs of longitudinal sections of human sperm heads after incubation for 5 h under capacitating conditions. (A): Immunoelectron microscopic peroxidase labelling on fixed/unpermeabilized spermatozoa incubated with monoclonal antibody pY20. Tyrosine phosphorylation (TP) immunoreactivity is localized in the anterior region of the head (detail is showed in the upper panel), external to the acrosome. (B): A swollen acrosome in which phosphotyrosine immunoreactivity was retained along the swollen membranes. (C): No immunoperoxidase labelling was detected on capacitated spermatozoa when the MOPC-21 antibody was used as a control for non-specific binding.

early event during capacitation. In five experiments on spermatozoa from different donors, the percentage of TP-positive spermatozoa increased significantly from zero time to 1 h of capacitation (Figure 3A). A further increase after 5 h of capacitation was not significant (Figure 3A). To determine the relationship between TP of the sperm head and acquisition of sperm-fertilizing ability, we assessed P-induced ARs and the P-enhanced HEPT under the same experimental conditions used to evaluate TP.

Analogous to the early increase in TP, a significant increase in P-induced ARs over the spontaneous rate was observed at 1 h of capacitation (Figure 3B); In contrast, at this time, sperm–oocyte fusion was as poor as in uncapacitated samples, but it was significantly increased after 5 h of capacitation (Figure 3C).

3.3 Effect of seminal plasma and a cAMP analogue

As shown in Figure 4A, in five experiments with different donors, the exposure of spermatozoa to 50% (v/v) seminal plasma during 5 h of capacitation prevented

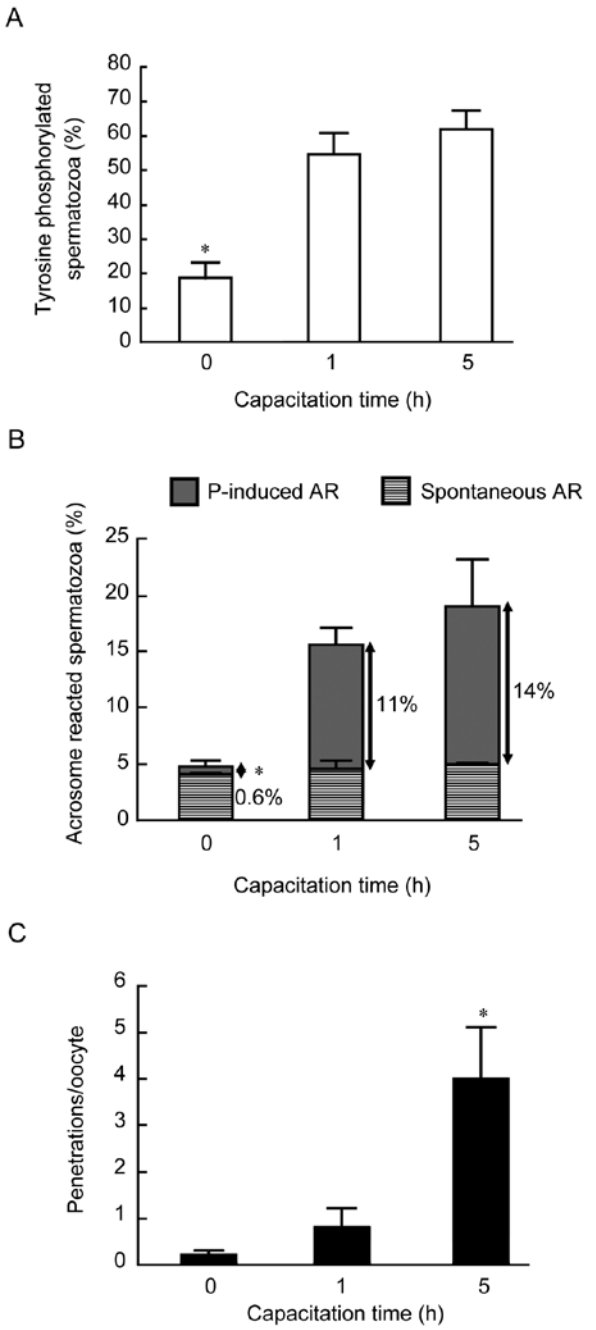

Figure 3. Time course of tyrosine phosphorylation (TP) of head proteins in human spermatozoa (A), spontaneous and progesterone (P)-induced acrosome reactions (ARs) (B) and P-stimulated sperm–oocyte fusion during capacitation (C). Results are from five experiments with different donor semen. (A): Overall significance: $P < 0.0001$ by ANOVA; $^{*}P < 0.05$, compared with 1 h and 5 h. (B): Overall significance: $P < 0.0001$ with ANOVA; $^{*}P < 0.05$, compared with 1 h and 5 h. (C): Overall significance: $P < 0.0001$ with PROC GLM. For this experiment 215 oocytes were used; $^{*}P < 0.05$, compared with 0 h and 1 h.

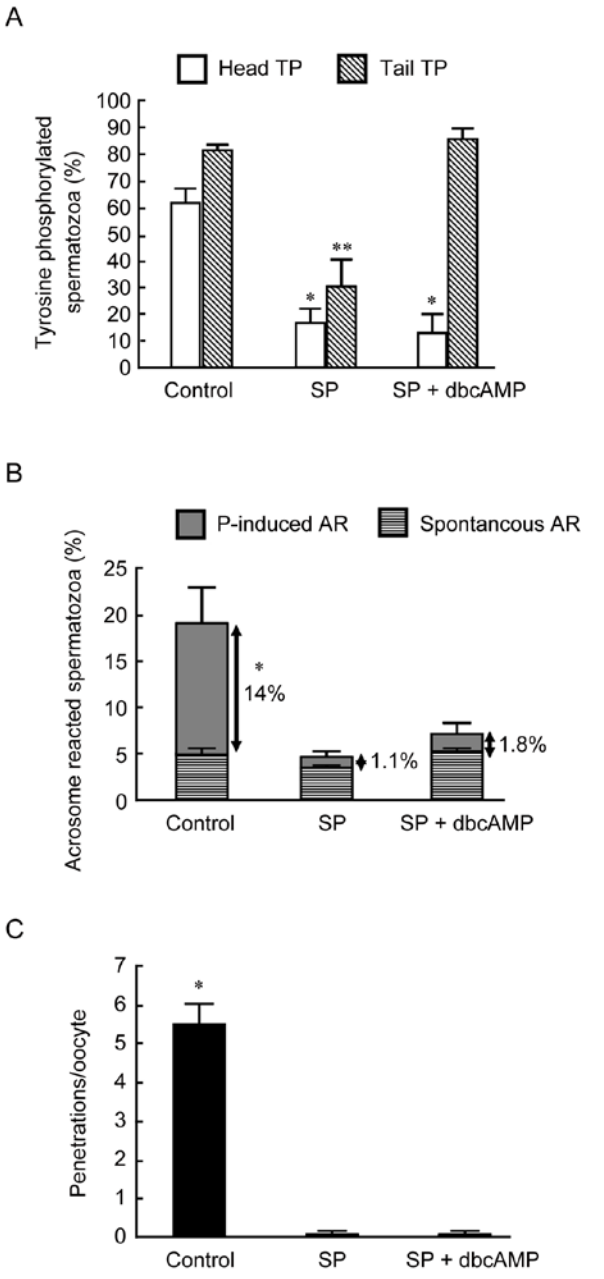

Figure 4. The effect of the presence of 50% (v/v) seminal plasma (SP) and SP with the cAMP analogue, dbcAMP (5 mmol L^{-1}), during sperm processing and during a 5-h capacitation on sperm head tyrosine phosphorylation (TP) (A), spontaneous and progesterone (P)-induced acrosome reactions (ARs) (B) and P-stimulated sperm–oocyte fusion (C). The results are from five experiments using different donor semen. (A): Overall significance: $P < 0.0001$ with ANOVA; $^{*}P < 0.05$, compared with the control and $^{**}P < 0.05$ compared with the control and SP + dbcAMP. (B): Overall significance: $P < 0.0001$ with ANOVA; $^{*}P < 0.05$, compared with SP and SP + dbcAMP. (C): Overall significance: $P < 0.0001$ with PROC GLM. For this experiment, 220 oocytes were used; $^{*}P < 0.05$, compared with SP and SP + dbcAMP.

the development of the capacitation-related increase in sperm head TP in formaldehyde-fixed/unpermeabilized samples. Seminal plasma also inhibited the increase in tail TP during the 5-h capacitation, as evaluated after fixation and permeabilization. The inhibition of head TP exerted by seminal plasma was not abolished by the concomitant addition of 5 mmol L^{-1} dbcAMP, which prevented seminal plasma inhibition of tail TP, as expected. In accordance with the cAMP/PKA pathway playing a minor role in head TP, the PKA inhibitor H89 (50 μmol L^{-1}) did not affect head TP immunoreactivity during a 5-h incubation under capacitating conditions (Figure 1G).

Seminal plasma inhibited both P-induced ARs (Figure 4B) and the number of penetrations per oocyte (Figure 4C). None of these effects was prevented by the concomitant addition of 5 mmol L^{-1} dbcAMP (Figures 4B and C).

4 Discussion

Although the capacitation-dependent tail TP in mammalian spermatozoa is a well-recognized event, TP of the sperm head is not as well characterized. TP of tail proteins accounts for the almost global TP occurring during capacitation, as detected by immunofluorescence assays [7] and immunoblotting [15]. However, as spermatozoa are highly polarized cells, the increase in TP of the sperm flagellum may have a role in the acquisition of hyperactivated motility during capacitation [11, 12], but not in the head events involved in fertilization.

An increase in the proportion of mouse and human spermatozoa undergoing TP of head proteins during capacitation has been reported by some authors [21–23] and denied by others [13, 17]. These conflicting reports could be explained by differences in the immunocytochemical methods used. In the present study, head TP immunoreactivity could be revealed only in 1% formaldehyde-fixed/unpermeabilized sperm suspensions. Aldehyde fixation ensures antibody access inside the cell by partially permeabilizing the sperm plasma membrane while still preserving membrane integrity, thereby avoiding the loss of internal membrane-bound antigens [27]. The permeabilization procedure is required for the consistent observation of TP immunoreactivity of the principal piece proteins, namely, the AKAPs, which are deeply localized, being the major structural fibrous sheath proteins of the principal piece [28]. Nevertheless, permeabilization and methanol fixation completely abolished head TP immunoreactivity, owing to their disruptive effects on sperm membranes and the loss of membrane-bound antigens. No TP immunoreactivity could be detected in unfixed capacitated human spermatozoa, which rules out any surface expression of phosphotyrosine residues. Reports have described a reduction in zona pellucida binding [20] and sperm–oocyte fusion [21] in live human spermatozoa pre-incubated with monoclonal anti-phosphotyrosine antibodies. More recently, a superficial TP immunoreactivity could be revealed in the head of live mouse spermatozoa using immunomagnetic beads [18]. Nevertheless, the authors, using the same method, failed to confirm any expression of phosphotyrosine residues in the human sperm head [19].

In the present study, head TP immunoreactivity was localized external to the acrosome, close to the cytoplasmic membrane when assessed by transmission electron microscopy that used a pre-embedding procedure and a sensitive peroxidase system. To our knowledge, this is the first report showing the application of the sensitive Envision + Dual Link Peroxidase System (DakoCytomation) for immunoelectron microscopy. A subsurface localization is inferred from the electron microscopy and the lack of immunoreactivity in unfixed, live spermatozoa.

We recently reported that flow cytometry is a rapid, simple and reliable technique to assess and quantify the levels of TP in fixed and permeabilized human spermatozoa [7]. As the use of fixed unpermeabilized human spermatozoa did not provide a clear dissociation between head and tail phosphotyrosine immunoreactivity, the interference of flagellar immunoreactivity prevented us from monitoring the dynamics of head TP during capacitation by flow cytometry. Immunofluorescence, however, did reveal an increase in TP of the human sperm head, similar to that reported for the flagellum [7], which seems to be an early event in the capacitation process with an ~ threefold mean increase within 1 h of capacitation.

Some interesting observations on the relationship between head TP and acquisition of sperm fertilizing ability arise from this study. Similar to the increase in head TP, the ability to display P-induced ARs was an early event during capacitation, whereas egg penetration occurred later, as expected [29]. These observations strongly suggest a direct link between the increase in head TP during capacitation and AR inducibility. In

streptolysin *O*-permeabilized human spermatozoa, a relationship between head TP and acrosomal exocytosis had been reported, as the inhibition of tyrosine kinase abolished both [30]. Intriguingly, a valosin-containing protein, known as p97 (VCP/p97), has been identified in the acrosomal region of human spermatozoa [23]. As in other cell types, tyrosine-phosphorylated VCP/p97 mediates the fusion of Golgi membranes and seems to be implicated in exocytosis processes [31], and its involvement in sperm preparation for ARs (a form of regulated exocytosis) has been hypothesized [23].

Seminal plasma inhibited the increase in head TP during capacitation, as well as P-induced ARs and oocyte penetration. None of these effects was prevented by the cell-permeable cAMP analogue dbcAMP. The inhibitory effect exerted by seminal plasma on capacitation-related events was expected, as many decapacitating factors from seminal plasma have been partially or completely characterized [6]. As seminal plasma is rich in cholesterol, it inhibits plasma membrane cholesterol efflux, thereby preventing an increase in sperm membrane fluidity, HCO_3^- influx and all other mechanisms involved in the activation of the cAMP/PKA pathway, a key event for capacitation and TP of the flagellum. Accordingly, on the basis of immunofluorescence, dbcAMP overcame the seminal plasma inhibition of tail TP, paralleling our recently published flow cytometric data of the whole sperm cell [7]. The involvement of the cAMP/PKA pathway in key functions of the flagellum is well known. In mammalian spermatozoa, cAMP/PKA-dependent phosphorylation of flagellar proteins is involved in the initiation and maintenance of sperm motility [32]. Early TP of AKAPs recruits the ubiquitous PKA to the fibrous sheath and facilitates additional local phosphorylation to enhance sperm motility [14].

The inability of dbcAMP to prevent the inhibition of head TP exerted by seminal plasma is a novel and interesting observation in the present study. In line with the cAMP/PKA pathway having a minor role in the phosphorylation of head proteins during capacitation, incubating sperm under capacitating conditions in the presence of the PKA inhibitor H89 did not affect the occurrence of head TP. Spermatozoa are highly polarized cells, and the cAMP/PKA pathway could have different regulatory roles in each subcellular sperm compartment. Sperm membrane receptors involved in the sperm–oocyte interaction undergo autophosphorylation, owing to an intrinsic tyrosine kinase activity triggered by receptor aggregation [21, 22], thereby making this process independent of the cAMP/PKA pathway. Indeed, functional aggregates could also occur spontaneously as a result of increased fluidity of sperm membranes during capacitation [22], thereby explaining the inhibitory effect of seminal plasma on head TP.

In conclusion, in human spermatozoa, head TP is a subsurface event that occurs early during capacitation, reflecting the dynamics of tail TP, the main determinant of global sperm TP, although a minor role is suggested for the cAMP/PKA pathway. Head TP also seems to be tightly related to acquisition of the ability of spermatozoa to display P-stimulated ARs, whereas the ability to fuse with oolemma and to decondense is a later [29] and more comprehensive capacitation-related event [33]. In this light, the occurrence of TP may be responsible for events occurring early during capacitation both in the tail (for example, hyperactivated motility) and in the head (for example, inducibility of ARs). However, it does not reflect the acquisition of the full capacitation-dependent sperm fertilizing ability.

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